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Systemic Protein Aggregation in Stress and Aging Restructures Cytoplasmic Architecture

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Systemic Protein Aggregation in Stress and Aging Restructures Cytoplasmic Architecture

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

September 2012

Dedication

Cytisus laburnum, simul vincet omnem

To my dad and mom who encouraged and enabled my education with countless sacrifices, I promised this graduation would be the one we would attend, and I am truly sorry I was not swift enough to make that possible.

Acknowledgements

Foremost, I thank my advisor Edward Marcotte, for not just a second lease on a life in science but one in an amazing lab environment. His intellectual rigor, enduring patience, amazing work ethic, and enthusiasm for discovery were an inspiration.

I thank my collaborators in this project: Gwen Stovall, Alice Zhao, Gabe Wu, and Mark Tsechansky for their comradery and support on this great adventure.

I thank the talented undergraduates: Maguerite West-Driga, Ariel Royall, and Tyler McDonald who stuck with me. Each of you will soon be a better scientist than I ever will, and I hope you enjoyed and learned from our research together nearly as much as I did.

Finally, I thank all the Marcotte lab members who moiled in the mines of systems biology with me. Many were the hours they sacrificed from their own projects to teach me, for that I am truly grateful. Thank you to Mark Carlson for teaching me cell culture techniques. Thank you to Traver Hart for introducing me to microscopy. Thank you to Kris McGary for the many discussions on the use of inductive and deductive reasoning in science (and a great many other fascinating topics). Thank you to Daniel Boutz for teaching me mass spectrometry. Thank you to Zhihua Li for teaching me the wonderful model system that is brewer's yeast. There was not a day in the Marcotte lab when I was not happy to be there.

Systemic Protein Aggregation in Stress and Aging Restructures Cytoplasmic Architecture

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The University of Texas at Austin, 2012

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A common maxim of protein biochemistry states, "structure is function." This is generally just as true for an individual polypeptide chains as for multi-protein complexes. The advent of yeast tagged-protein libraries has allowed systematic screening of a protein's local interaction partners as well as a roughly mapping its cellular location. Recently our group and others discovered hundreds proteins forming new structures in stationary phase yeast cells using the yeast GFP-tag library. That equates to well over a quarter of normally diffuse cytoplasmic proteins assembled into discrete structures that appear as foci or fibers, all of unknown function. This study provides evidence that many of these foci are formed by protein aggregation- that contrary the maxim, structure can be dysfunction. Furthermore, this study uses yeast to demonstrate the generality of cytoplasmic protein aggregation in response to a variety of stresses, provides evidence that increasing aggregation of particular cytoplasmic proteins correlates with aging even across organisms, and proposes a theoretical framework for how cellular energy levels affect protein aggregation propensity.

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Chapter 1: Introduction

My interest and involvement in the research described in this dissertation began following a talk by Gwendolyn Stovall were she described an astounding phenomenon discovered by Rammohan Narayanaswamy. He and fellow lab mates had been screening the yeast GFP-tagged strain library to track the intracellular location of proteins under various conditions. In a screen of this library grown to stationary phase he found hundreds of new cytoplasmic bodies. An astounding 30% of normally diffuse cytoplasmic proteins formed foci bodies or fibers, representing a massive restructuring of the yeast cytoplasm including much of central metabolism. However, far from being a unique occurrence, these structures are just the latest in a growing assortment of intracellular bodies formed by metabolic enzymes.

In fact, observations of such bodies have increased markedly in recent years. The technological capacity to perform large-scale microscopy screens of protein localization has made possible cell biological studies that focus on particular cellular conditions. Such screens have been further abetted by the availability of extensive libraries of bacterial, yeast, and mammalian cells expressing proteins fused to reporters. In parallel, biologists and bioengineers have begun constructing novel multi-enzyme complexes in order to alter or improve metabolic capacity. Thus, the understanding of the cellular principles underlying the assembly of large protein bodies and their contribution to the dynamic regulation of metabolism is ripe for exploration and exploitation.

What follows is a brief background on intracellular protein bodies formed by metabolic enzymes. We discuss the current understanding of when and why such bodies form, and what their formation implies for the functionality—and dysfunctionality—of the enzymes that comprise them. We then describe several of the foundational experiments, as published by Narayanaswami et al. 2009 [1], as essential context for the remaining chapters.

This chapter has, in part, been excised and reworked from a review by O'Connell et al. 2012 [107].

TYPES OF INTRACELLULAR BODIES

There are a wide variety of intracellular bodies, but they can be roughly classified based on their composition and structures. Bacteria for the most part (although exceptions exist) do not have membranous compartments, and thus their intracellular bodies tend to be almost exclusively proteinaceous, and to serve as sub-cellular compartments with specialized interiors optimized for their relevant biological roles. Originally called polyhedral bodies when discovered in electron micrographs of *P. uncinatum* in 1956, bacterial microcompartments are icosahedrons ~100-200 nm in diameter delimited by a 3-4 nm thick protein shell [2].

In eukaryotes the formation of membranous compartments is more the norm, and beyond mitochondria and chloroplasts a profusion of specialized intracellular metabolic compartments have been discovered in recent years. These compartments bear a particular relevance to studies of self-assembly of metabolic enzymes, as they frequently

exhibit a high level of enzyme self-organization. It can be argued that the crystalline or quasi-crystalline organization of at least some metabolic enzymes within intracellular membranous compartments is a direct result of their increased concentrations within these microbodies.

Finally, beyond the microcompartments and microbodies discussed above, individual metabolic enzymes have been observed to form intracellular fibers and foci. Textbook cases for which the fiber is known to be the enzymatically active form include acetyl coA-carboxylase and β-glucosidase. Many additional examples of fiber-forming metabolic enzymes have been recently identified, but their functionality is as yet unestablished.

Bacterial microcompartments

The archetypal bacterial microcompartment, the carboxysome, earned its name and association with carbon fixation in 1973 when the CO_2 fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) copurified with polyhedral bodies from the aerobic sulfur bacterium *H. neapolitanus* [3]. Initial evidence (such as dispersal upon revival/re-feeding [4]) supported the idea that carboxysomes were storage bodies rather than the major sites of carbon fixation. However, studies over the next twenty years showed that the RuBisCO in carboxysomes was active, and that carboxysomes also contained carbonic anyhdrases to convert bicarbonate into CO_2 [5], [6].

Crystal structures of carboxysome shell proteins model the wall as a single layer of interlocking hexagonal subunits with a pore in the center of each. The sheets of hexagonal subunits are joined at the vertexes by pentagonal subunits to complete the icosahedrons [7], [8]. The shell seems to provide selective permeability to metabolites, while blocking the diffusion of even smaller gas molecules by an unknown mechanism [9]. The co-localization of enzymes and substrates within the diffusion barrier of the carboxysome shell greatly increases the efficiency of carbon fixation, and carboxysomes are the main sites of CO_2 capture in cyanobacteria.

Searches for sequence homology to the major shell proteins have revealed that at least 189 bacterial species contain shell protein orthologs in gene clusters that potentially produce microcompartments [10]. Among these, microcompartments with functions other than CO_2 capture have begun to be characterized. In *E. coli* and some *S. enterica* serovars orthologs of the carboxysome shell proteins were found in operons containing genes essential for growth on ethanolamine [11]. Acetylaldehyde, is a toxic and volatile intermediate in this pathway, and might otherwise rapidly diffuse away in the absence of the shell proteins. Thus, a portion of the ethanolamine degradation pathway occurs within an ethanolamine utilizing microcompartment (EUT) microcompartment [12]. A similar 1-2 propanediol utilizing (PDU) microcompartment was found in an *S. enterica* serovar (*Typhemurium)* [13]. The PDU microcompartment may shield the cell from the toxic intermediate propionaldehyde [14], [15]. The discovery of an N-terminal targeting sequence for loading proteins into PDU microcompartments and the transgenic

expression of functional carboxysomes containing fusion proteins in *E. coli* mark the first steps toward rationally engineering bacterial microcompartments [16], [17].

Aggregates within microbodies

It is interesting that protein microcompartments are primarily found in bacteria, rather than eukaryotes, in the same way that it is interesting that bacteriophage primarily have protein coats, while their eukaryotic counterparts have membranous coats. It may be that as the complexity of a system scales, the opportunities for inadvertent protein mis-aggregation also scale (a point we will touch on later), and that the less precise organization of lipids relative to proteins is a hedge against such aggregates. Conversely, as suggested above, enzyme compartmentalization may provide opportunities for increasing concentrations to the point where ordered aggregation is possible. Whatever the reason, eukaryotes frequently compartmentalize metabolic enzymes within membranes. We highlight three cases of ordered aggregates within peroxisomes as examples typical of bodies seen in other membrane bound compartments such as the mitochondria [18] and the chloroplast [19], [20].

Peroxisomes are eukaryotic microbodies bound by a single lipid membrane. These structures, typically 100 nm to 1 μm across, compartmentalize enzymes and substrates at high concentrations for particular metabolic pathways to improve the rates of catalysis or to shield the rest of the cell from the potential damaging effects of reactive intermediates.

Most peroxisomes possess one or more enzymes for purine catabolism or salvage, usually xanthine oxidase and urate oxidase. These proteins are often clustered, even to the extent of forming amorphous and crystalline inclusions. Urate oxidase is a cuproprotein that normally forms homotetrameric rings, but in some mammalian peroxisomes these tetramers stack into fibers, which combine into crystalline cores [21]. These crystalline cores are a common feature of many types of peroxisomes, though their effects on the enzymatic activity are unknown.

In plants, peroxisomes commonly specialize in ß-oxidation, as well as enzymes to remove hydrogen peroxide produced by the oxidases of ß-oxidation. Electron microscopy of plant peroxisomes reveals crystalline and amorphous inclusions, likely composed of catalase, a homotetrameric enzyme that degrades hydrogen peroxide [22]. Biochemical studies comparing crystalline catalase to diffuse catalase found that the crystalline catalase had up to 10-fold less specific activity but greater stability under UV, pH, and temperature stresses [23]. While there is a clear loss of function due to aggregation, the gain of structural stability in a highly oxidizing environment could be an example of adaptive change in catalytic potential mediated by forming larger aggregates.

Specialized types of peroxisome, called Woronin bodies, are found in filamentous fungi and staunch the flow of cytoplasm from hyphal wounds [24].The major and essential component of Woronin bodies is an aggregate of HEX protein oligomers

forming the eponymous hexameric crystalline cores. HEX proteins are most closely related in structure and sequence to eIF-5a, though the residues responsible for inter-subunit contacts are different and the precise enzymatic function is unknown (Yuan et al. 2003). Mutating residues at the oligomerization interface abolishes both the wound healing function and the classic polymerization phenotype, showing this to be an example of a functional aggregate [25].

In mammals the processed form of the ß-lactamase-like protein (LACTB) polymerizes into ordered filaments hundreds of micrometers in length in the intermembrane space of mitochondra [18]. It is unknown what role these fibers have within mitochondria, nor what drove the evolution of fiber formation from the non-fiber forming bacterial paralog.

FIBERS AND FOCI

While microcompartments and membranous organelles are complex, highly structured organizing centers of metabolism, metabolic enzymes also self-assemble into a wide array of simpler intracellular bodies, among them fibers and foci. Many such bodies have been discovered— some are clearly functional; the functions of others have yet to be established. It can be argued that the probability of forming functional aggregates scales with the concentrations of the enzymes involved, and thus it may be unsurprising that a large number of these fibers and foci seem to be enzymes that support key

metabolic processes, such as carbon utilization, nitrogen fixation, and nucleotide biosynthesis.

Fibers of metabolic proteins: carbon utilization

Self-assembling fibers of a single enzyme are perhaps the simplest metabolic bodies observed in cells. The textbook example of a functional fiber is acetyl-CoA carboxylase; it was the first and remains the best characterized of all enzymatic fibers. Most mammals encode acetyl-CoA carboxylase as a single multi-domain protein, nominally a homodimer, but which can assemble into polymers with >50-fold greater activity [26], [27]. In mammals, there are two isoforms of the enzyme, one localized to the cytoplasm and the other to the mitochondria. Both isoforms carry out the first and rate-limiting step of fatty acid biosynthesis by carboxylating acetyl-CoA to malonyl-CoA. While polymerization is dependent on enzyme concentration, there are also a number of other mechanisms that shift the enzymes between inactive monomers and the active polymer form [28]. Phosphorylation / dephosphorylation will decrease / increase polymerization, respectively; allosteric binding of citrate induces polymerization, while excess product (malonyl-CoA) triggers depolymerization; polymerization can also be induced by MIG12 binding [29]. For the mitochondrial isoform MIG12 binding alone is sufficient to drive polymerization, and reduces the concentration of citrate needed for polymerization ~5-fold for the cytoplasmic isoform [29].

Fibers of β-glucosidase were first reported in electron micrographs of oat plastids in 1965 [30]. This enzyme is nominally a homohexamer that hydrolyzes β1→4 glucose bonds; it

also cleaves avencosides as an anti-fungal defense. Forty years later, structural studies revealed rings of hexamers stacked into fibers up to 2 μm in length [31], and biochemical analyses provided evidence that the fibers might be the active form of the enzyme: longer fibers were more active in hydrolyzing avencosides and more resistant to inhibitors than shorter fibers [31].

Fibers of metabolic proteins: nitrogen utilization

Several enzymes at the core of nitrogen metabolism also form fibers. Studies of *E. coli* glutamine synthetase (Class I), which catalyzes the ATP-driven addition of ammonia to glutamate, found that zinc progressively induces the dodecameric rings of the enzyme complex to aggregate into fibers [32]. Mammals have an eye-specific glutamine synthetase (Class I-like) that is catalytically inactive but essential for proper eye development [33], one of a number of chance adaptations of structured enzyme aggregates to serve as eye proteins, such as crystallins. In yeast, glutamine synthetase (Class II) is a decamer that forms foci *in vivo* under starvation conditions, although its ultrastructure remains to be determined [1]. When cells are starved of glutamine, they degrade glutamate to α-ketoglutarate and ammonia using the NAD-dependent glutamate dehydrogenase in mitochondria. In mammals, this enzyme normally forms homohexamers but at high concentrations associates into either highly ordered filaments or helical fiber bundles [34]. It is not clear what effect polymerization has on enzyme activity [35]; however, glutamate dehydrogenase has a host of allosteric regulators, including zinc, ADP, and GTP, and those that activate function also promote

polymerization and decrease thermal aggregation [36–40]. The reverse reaction—glutamate synthesis—can be carried out either by highly related enzymes using NADPH as a cofactor, or by glutamate synthase, which carries out a transamination from glutamine to α-ketoglutarate forming two molecules of glutamate. In starved yeast, the GFP-tagged glutamate synthase forms fibers [41]; like glutamate dehydrogenase, the enzyme forms homohexamers, and the fibers observed by fluorescence microscopy may represent fibrillar bundles of the homohexamers.

Fibers of metabolic proteins: nucleotide biosynthesis

Cytidine triphosphate (CTP) synthase

Recently, multiple groups have reported that CTP synthase forms filamentous and ring structures *in vivo* in the fly [41–43], bacteria [44], budding yeast [41], rat [41], and human cells [43], [45]. In the crescent-shaped bacterium *C. crescentus*, CTP synthase forms a single rod lining the inner curvature of the cell. In *Drosophila*, CTP synthase filaments, termed cytoophidia (meaning "cell snake"), exist in two forms, coined "micro-cytoophidia" and "macro-cytoophidia," the latter being considerably thicker and longer than the former. In yeast, CTP synthase assembles into filaments as cells enter quiescence upon culture growth to stationary phase. In fact, yeast cells express two CTP synthase homologs, Ura7p and Ura8p, that co-localize within the same filaments, but not with other known filament-forming proteins [41].

Initial data suggest that these fibers may be due to the self-association of CTP synthase, and do not require energy or special machinery (such as chaperones or scaffolds) for formation. Filaments are seen to form upon heterologous expression of *C. crescentus* CTP synthase in *E. coli*, and native *E. coli* CTP synthase forms filaments both *in vivo* and *in vitro* [44]. That said, a number of studies now implicate additional components in the fibers.

While the filaments show distinct intracellular locations, these are apparently different in different organisms. In *C. crescentus*, CTP synthase filaments have been shown to co-localize to crescentin (CreS) along the inner curvature of the cell and apparently help regulate the crescent cell shape [44]. In rat neurons, CTP synthase filaments occurred in axons but not dendrites [41]. Fly micro-cytoophidia were found to associate with Golgi bodies and in some cases with microtubules (Liu 2010). In contrast, human and yeast CTP synthase filaments were not observed to co-localize with microtubules [41], [45], and human CTP synthase filaments were also not observed to co-localize to Golgi bodies or centrosomes and were not enriched in actin or vimentin [45].

Compounds that modulate CTP synthase protein function modulate fiber formation. For example, CTP synthase inhibitors such as Acivicin and the glutamine analogs 6-diazo-5-oxo-L-norleucine (DON) and azaserine [43], [45] have dramatic effects on CTP synthase filaments. Interestingly, CTP synthase inhibitors produce different effects in different organisms: DON treatment disrupts CTP synthase filament

formation in *C. crescentus,* and also disrupts filaments of heterologously expressed *C. crescentus* CTP synthase in *Schizosaccharomyces pombe* or *E. coli* [44]. In contrast, DON and azaserine treatment promote filament formation in fly cells, and DON and Activin treatment induce filament formation in human cells [43], [45]. Notably, DON binding induces tertiary [46] and quaternary [47] structural changes in *E. coli* CTP synthase. As in the glutamine synthetase case mentioned above, such allosteric conformational changes also alter the conformational state of neighboring subunits coordinately [48], which is consistent with the hypothesis that differences in intersubunit amino acid contacts might be exposed or hidden by conformational changes. This would not only help to explain the evolution of fiber formation, but also how fiber formation is functionally related to regulatory logic.

Filament formation can also vary broadly according to systemic conditions, including cell types, stages, and growth conditions. Ingerson-Mahar et al. reported that mCherry-CTP synthase filaments were generally shorter in newly formed stalked cells of *C. crescentus*, and then elongated with the progression of the cell cycle; subcellular localization was also dependent on cell cycle [44]. The opposite was observed in human cells, where expression of filaments occurred in all phases of the cell cycle in HEp-2 cells [45], and a similar ubiquity was found in HeLa cells [43]. However, in other human cells, the filaments varied by cell type and culture conditions. For example, undifferentiated, uninduced human embryonic stem cells contained CTP synthase rings and rods, but lost them when stimulated by retinoic acid to differentiate [45]. Similarly, filaments of the

yeast CTP synthase Ura7p are strongly induced by growth to saturation or glucose depletion, but disappear upon addition of fresh medium. Thus, CTP synthase fibers currently represent something of a quandary—while widely observed, they show mixed regulatory logic across organisms, and their enzymatic functionality has yet to be established.

Inosine 5'-monophosphate dehydrogenase (IMPDH)

In 2011, a study in human cells demonstrated that cytoplasmic filaments consisting of human CTP synthase also contained IMPDH [45]. IMPDH catalyzes the NAD-dependent oxidation of inosine monophosphate (IMP) to xanthosine monophosphate (XMP), the first and rate-limiting step for the synthesis of guanosine nucleotides. The predominant isotype, IMPDH type II, is highly expressed in neoplastic and differentiating cells, making it an inviting target for antiproliferation drugs, such as the immunosuppressive noncompetitive inhibitor mycophenolic acid (MPA) [49], [50]. MPA induced IMPDH rings and filaments in cultured human cells, reduced the enzyme's specific activity in cell lysates, and induced purified IMPD homotetramers of ~15nm in diameter to form large, disordered aggregates *in vitro* [51]. Both MPA-induced rings and fibers *in vivo* and aggregation *in vitro* could be dispersed by the addition of GTP at physiological concentrations, with GTP addition restoring the activity of MPA-inhibited IMPDH in cell lysates [51]. These results are consistent with a hypothesis that MPA stabilizes inter-tetramer interactions, shifting the equilibrium between active IMPDH tetramers and inactive fibers to favor the inactive form. These results also beg the

question if cells might naturally form IMPDH fibers in order to store or regulate the enzyme in inactive form rather than destroying or recycling it.

Purinosomes

Although sampling may be biased by history, the enzymes of nucleotide metabolism appear to be particularly prone to form intracellular bodies. In particular, the existence of a multi-enzyme complex consisting of the all the members of the *de novo* purine biosynthesis pathway, termed the "purinosome," has been postulated for some time based on the accumulation of a variety of evidence from many experiments. The *de novo* purine biosynthesis pathway encompasses a ten-step enzymatic reaction converting phosphoribosyl pyrophosphate (PRPP) to inosine monophosphate (IMP). In higher eukaryotes such as mammals, *de novo* purine biosynthesis is carried out by phosphoribosyl pyrophosphate (PPAT), the trifunctional phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, and phosphoribosylaminoimidazole synthetase (GART), phosphoribosylformylglycinamidine synthase (FGAMS), the bifunctional phosphoribosylaminoimidazole carboxylase and phosphoribosylaminoimidazole succinocarboxamide synthetase (PAICS), adenylosuccinate lyase (ADSL), and the bifunctional 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC). The *de novo* purine biosynthesis pathway is up-regulated when exogenous hypoxanthine is unavailable [52] [53].

In higher eukaryotes these ten enzymes have fused into 6 polypeptide chains, three of which possess multiple active sites as listed above. Perhaps the most interesting of these is the trifunctional protein GART, which catalyzes the second, third, and fifth enzymatic steps in the pathway. Early hints that the purinosome might form included co-immunoprecipitation of trifunctional GART and bifunctional PAICS from chicken liver [54] along with serine hydroxymethyl transferase and the trifunctional methylene tetrahydrofolate dehydrogenase (MTHFD1). These latter two enzymes form a cycle for the production of the labile 10-formyl tetrahydrafolate coenzymes required for steps 3 and 9 of purine biosynthesis. Cross-linking studies *in vitro* found a physical interaction between GART and MTHFD1, and their interaction increases GART activity [55]. Fluorescence microscopy of transiently-expressed, fluorescently-tagged proteins showed colocalization between tetrahydroflolate synthase and both FGAMS and GART.

In 2008, the Benkovic group reported the first direct observation of the purinosome in cells [56]. Fluorescent protein constructs of the six mammalian purine biosynthesis enzymes formed punctate intracellular foci when transiently over-expressed in HeLa cells in purine-depleted culture medium. FGAMS-GFP was shown to co-localize with the five other enzymes of the pathway, suggesting the assembly of *de novo* purine biosynthesis enzymes into purinosomes. FGAMS-GFP foci could be dissolved by exchanging purine-depleted for purine-rich medium, although the addition of the purine hypoxanthine to purine-depleted medium did not dissolve the foci. Intracellular bodies that included PPAT, GART, ADSL and ATIC were also detected via

immunofluorescence confocal microscopy of endogenous enzymes in a number of human cell types, including primary kerotinocytes. The bodies formed and dispersed when culturing cells in purine-depleted or purine rich medium, respectively [57].

Inhibitor studies have yielded some insights into the mechanism of purinosome formation. Addition of the microtubule-disrupting agent nocodazole reduced purinosome foci formation and decreased overall cellular purine synthesis [58], suggesting that the formation of these bodies may require cytoskeletal directed active transport. Casein kinase 2 (CK2) inhibitors 4,5,6,7-tetrabromo1H-benzimidazole and 2-dimethylamino-4,5,6,7-tetrabromo-1H-bezimidazole induced foci formation by FGAMS-GFP, GART-GFP, or PPAT-GFP, while the subsequent addition of a different CK2 inhibitor, 4,5,6,7-tetrabromobenzotriazole, appeared to reverse or suppress foci formation. In addition, while CK2 inhibitors affected FGAMS-GFP, GART-GFP, and PPAT-GFP foci formation, similar effects were not seen on PAICS-GFP, ASL-GFP, and GFP-ATIC unless each was co-transfected with one of the former three proteins tagged with orange fluorescent protein (OFP)([58]). These results suggest a possible, but as yet unclear, role for CK2 in purinosome regulation. One possibility is that phosphorylation by CK2 disperses assemblies, as seen for acetyl CoA-carboxylase [59].

The study of the purinosome is also a case study in how the methods used to query intracellular bodies potentially influences outcomes. The visualized purinosome clusters have largely been observed by transient over-expression. In contrast, previous

efforts using cells stably transfected with purinosome constructs did not yield visible purinosome bodies [60]. Overexpression of proteins above their native levels has been shown to result in their aggregation [61–65], and the transiently overexpressed purine biosynthetic enzymes form intracellular bodies to extents that correspond to each enzyme's predicted aggregation propensity [66]. The resultant bodies are marked by ubiquitin and heat shock chaperones, suggesting that they may represent aggregated protein clusters [66].

The formation of functional purinosomes is not necessarily inconsistent with the observation of aggregated purine biosynthetic enzymes. Indeed, both may be true, depending on the cell state and method of observation. Purinosome bodies appear to be quite heterogeneous, as the penetrance of fluorescent body formation varied broadly for each individual enzyme, ranging from 5-77% of the cells [56]. A similar broad distribution was observed for CK2 inhibitor-mediated effects (ranging from 15-95% penetrance) [67]. Regulatory influences can also be interpreted in terms of either functional or non-functional aggregation (or both): while microtubules and CK2 could regulate purinosomes, they have also been implicated in general protein aggregation [68], [69]. Nocodozole inhibition of purinosome formation [70] is consistent with functional assembly requiring microtubules, but nocodozole treatment also inhibits inclusion body formation of aggregated huntingtin [68], [71]. Future studies will clearly be required to address the key questions of functionality of the purinosome bodies, ideally leading to purification and *in vitro* characterization, and to assess the relationships, if any, between

the intracellular bodies formed by overexpression of purine biosynthetic enzymes and the endogenous forms of those proteins.

LARGE-SCALE SCREENS REVEAL MANY ADDITIONAL FOCI AND FIBERS

The frequency at which intracellular bodies have been found during the study of metabolism either indicates that metabolic enzymes are prone to aggregation, or perhaps that all enzymes are prone to aggregation and, to date, biochemists just happen to have largely studied metabolic enzymes. In fact, large-scale microscopy screens of protein localization dynamics have revealed tens to hundreds of additional enzymes that form intracellular bodies. Using cell microarrays, Narayanaswamy *et al.* surveyed large-scale trends in yeast (*S. cereviseae*) protein reorganization using a genome-wide green fluorescent protein (GFP) fusion library [72]. When ~800 yeast strains expressing normally cytosolic GFP-tagged proteins from their native locus in the genome were grown to stationary phase, 180 proteins involved in intermediary metabolism and stress response were observed to form punctate cytoplasmic foci. The formation of a number of these proteins was also confirmed by immunofluorescence and mass spectrometry of untagged proteins, with 33 proteins confirmed by both microscopy and mass spectrometry [1].

A second screen of a portion of the *S. cereviseae* GFP library by Noree *et al.* found 27 additional foci structures (Noree et al. 2010). Of these, three were metabolic proteins that form fibers. One was a CTP synthase fiber (discussed above). The other two

fibers, composed of Psa1 and Glt1p, were entirely new. Psa1p is a GDP-mannose pyrophosphorylase essential for building the glycoprotieins of the cell wall and is highly conserved across eukaryotes. The other, Glt1p (also discussed above), was a NAD-dependent glutamate synthase, which along with Gln1p forms one of the core ammonia incorporation pathways. It is currently unclear whether these fibers are functional and if so what role they might serve.

There have also been several systematic microscopy screens of protein localization performed in the fission yeast, *Schizosaccharomyces pombe* [73–76]. While these screens were not specifically searching for new structures, they still describe a number of proteins as existing as cytoplasmic 'dots' (e.g., CTP synthase and Ade4p, as was also seen in *S. cerevisiae* [1]) or in fibrous morphologies (e.g., Pil1p, an essential cell wall peptidoglycan synthetase).

A localization study of ~300 cytoplasmic proteins in *Caulobacter crescentus* by Werner and colleagues identified a number of proteins that showed non-diffuse localization [77]. Of these, they described 29 proteins' cellular distributions as foci, 129 proteins as patchy/spotty, and 3 as filament-forming. Two of the filament-forming proteins are CTP synthase and an associated structural protein CreS. The other fiber is formed by UDP-N-acetylmuramate-alanine ligase. Similar to Psa1p, it is an essential enzyme for peptidoglycan synthesis.

These large-scale screens clearly reveal a remarkably extensive assortment of intracellular bodies forming across diverse environmental conditions. It would appear that at least some of these bodies are metabolically inducible and form reversibly, strongly suggesting functionality. For example, the yeast purine biosynthetic enzyme Ade4-GFP formed foci in the absence of adenine, and cycling between punctate and diffuse phenotypes could be controlled by adenine subtraction and addition. Similarly, yeast glutamine synthetase (Gln1-GFP) foci cycled reversibly in the absence and presence of glucose [1]. Finally, there is good evidence for the yeast translation initiation regulatory complexes eIF2 and eIF2B existing as polymerized fibers during log phase growth [41]. As this is precisely when translation rates are the highest in yeast, it argues the fibers may be functional and regulated. However, as with the purinosome, caution must be used in interpreting whether the bodies form for functional roles, or whether regulated changes in concentration inadvertently lead to intracellular aggregates.

Most large-scale localization studies use fusion proteins, and the properties of the tag can affect the solubility and interactions of the tagged protein. One recent study found that a commonly accepted body formed by Clp proteases was entirely dependent on certain fluorescent tags for formation. In the absence of a tag or using a GFP evolved for monomeric expression in *E. coli*, Clp proteases did not form the bodies. The authors propose that the fluorescent protein tags dimerize, causing homo-oligomeric complexes to assemble into an extended network and producing an intracellular body [78]. If this is a major cause of intracellular bodies, it predicts the set of foci forming proteins should be

strongly enriched for oligomers. Thus structures discovered by large-scale fluorescence localization screens need to be confirmed by orthogonal, preferably tag-free, methods to verify their biological relevance.

WHAT'S GOING ON? THREE POTENTIAL ROLES FOR INTRACELLULAR BODIES

The panoply of intracellular protein bodies also raises interesting questions regarding their evolution and maintenance within cells. We conclude by offering additional speculations on several particular aspects of how and why such bodies might evolve.

Case studies such as CTP synthase and the purinosome raise many interesting questions regarding the functionality of intracellular bodies. It is thus useful to consider why metabolic enzymes might assemble into such large intracellular assemblies. In general, metabolic enzymes are notable for often forming complex quaternary structures, some larger (e.g. pyruvate dehydrogenase) than even the largest single proteins. It is possible that these massive intracellular assemblies provide functional advantages to the cell, such as catalytic efficiency or improved regulation. Alternatively, these structures might be depots for the storage of functional proteins or the disposal of dysfunctional ones. While we have seen that numerous metabolic enzymes clearly form functional and well-characterized meta-assemblies, it is not yet clear for many of the most recently discovered intracellular bodies whether they represent functional bodies, storage bodies,

or aggregates. Distinguishing these roles represents one of the major challenges for studying these structures.

Catalytic efficiency and improved regulation

Classically, enzymes have been thought to organize into multi-subunit assemblies in order to improve their functionality. Quaternary structures enable the channeling of substrates between active sites on individual subunits, thereby protecting labile intermediates from side reactions in the cell, or protecting the cell from toxic reaction intermediates. For example, the first intermediate substrate in *de novo* purine biosynthesis, 5-phosphoribosylamine, has a cellular half-life of 38 seconds, and channeling is essential for its subsequent coupling to glycine [79]. Also the addition of CO_2 to aminoimidazole ribonucleotide to form 4-carboxy-5-aminoimidazole ribonucleotide occurs without the use of biotin or ATP. In plants, bacteria, and yeast this occurs through two enzymatic steps with N^5-CAIR as an unstable intermediate substrate, perhaps driving the fusion of the enzymes responsible for these two steps in yeast and plants. Finally, the 5,10 formyl methylene tetrahydrofolate coenzyme used in steps 3 and 9 is moderately labile, with a half-life of 30 min [55]. Such observations underpin the search for the purinosome, which would in principle localize the enzymes within sufficient proximity to prevent the diffusion of unstable intermediates or substrates. Similarly, the peroxisome shields cells from peroxide radicals generated by oxidases in fatty acid and purine catabolism. A related effect is seen in the formation of ethanolamine-utilizing microcompartments that shield cells during growth on

ethanolamine by localizing the production and degradation of toxic aldehydes within a protein shell.

Channeling substrates between active sites in a quarternary structure or within a compartment can also greatly improve metabolic efficiency even when no side reactions are in play. For instance, carboxysomes prevent the diffusive loss of CO_2 during carbon fixation and thereby allow unicellular organisms to achieve C4 plant-like efficiencies. In an even grander organization of metabolic machinery, the cellulosome anchors enzymes involved in cellulose production via elaborate, interlocking multidomain protein scaffolds on the cell surface.

Finally, quarternary structure formation allows regulation by cooperative interactions and allosteric effectors. There are of course many such known examples of cooperativity in enzyme oligomers. Among these, the dodecameric glutamine synthetase from *E. coli* is one of the best understood. In addition to two covalent modification enzyme systems, glutamine synthetase has eight direct allosteric inhibitors that individually partially inhibit activity and together cooperatively inhibit activity [80]. The allosteric inhibitors bind active sites that are positioned at the interface between the subunits of the two hexameric rings, and in this way binding is transduced into structural changes that can be transmitted between enzymes in both rings. This mode of regulation effectively integrates information on the metabolic state of the cell with overall enzyme

activity [81], and such changes could in principle modulate the formation of glutamine synthase foci and fibers *in vivo*, although such an effect has yet to be shown.

Storage depots

It has also been hypothesized that proteins may assemble into macromolecular 'depots' in which individual components can be transiently held and released. The advantage of such depots is that proteins need not be resynthesized but are instead retained for potential future use, especially in conditions where rapid redeployment may be required. For example, in the stationary (quiescent) phase, cells have a remarkable ability to weather extreme stress conditions and yet can rapidly re-enter the cell cycle [82]. Many changes accompany the transition into stationary phase. Stationary phase yeast cells, for example, exhibit decreased metabolic rates, increased size and density, and in general cease proliferating. The cell walls increase in thickness to provide osmo and thermotolerance, and the cells accumulate intracellular carbohydrates, including glycogen and trehalose, which may serve to help protect the cells against a variety of stresses [82]. Although rates of transcription and translation are dramatically decreased in quiescent cells as compared to exponentially growing cells [83], [84], the quiescent cells must remain able to rapidly restart growth when nutrients do become available. Quiescent cells have been shown to maintain available pools of important cellular components in forms that can be mobilized quickly, including cytoplasmic processing bodies (P-bodies) containing mRNAs that can be translated upon restarting growth [85], and ‘actin bodies’—localized accumulations of actin that can reassemble into actin fibers and

patches as necessary when cellular growth restarts [86]. Overall, quiescent cells appear to be rich with dynamic depots that are important for re-entry into the mitotic cell cycle. Such a trend is consistent with the tendency for many cellular proteins to be organized—both spatially and functionally—in a manner consistent with the needs of the cell.

Aggregation of dysfunctional and/or unfolded proteins

In contrast to the above examples of intracellular bodies with active functional roles or storage of functional potential, a third major category of intracellular bodies is now well established—those composed of aggregated and possibly dysfunctional unfolded proteins. Such bodies often involve active cellular processes for collecting, sequestering, and disposing of the aggregates [87], but can also form when high levels of expression trigger self-association and aggregation [88]. Aggregated proteins are often sequestered, often via active transport along the cytoskeleton, to specific cellular sites, such as e.g. aggresomes [89], IPOD, and JUNQ [90].

The best characterized examples of protein aggregates and intracellular aggregation bodies include amyloid fibers and foci, and inclusion bodies. Amyloid fibers, in particular, are linked to a range of human diseases including Alzheimer's, Parkinson's, and Huntington's [91], but also occur broadly across proteins and organisms [92], [93]. The toxicity of protein aggregates is generally attributed to not only the depletion of functional machinery [94], but also to the creation of pores inside cell membranes by small oligomers [95]. At the early stages of aggregation, small oligomers of amyloid

aggregates have structural similarities to pore-forming bacterial toxins and eukaryotic pore proteins [96], and their insertion into membranes leads to ion loss and cell death [97]. Both yeast and human prions constitute a continuous spectrum of aggregation with multiple morphologies [98], [99], and aggregated prion protein seeds can propagate readily to daughter cells [100].

FOUNDATIONAL EXPERIMENTS

S. cerevisiae GFP-strain library

Researches in the lab of Erin O'Shea constructed the library with the goal of measuring the location of all the proteins inside a yeast cell. To create the library PCR products containing a short linker sequence followed by GFPS35T and a selectable marker gene were inserted at the C terminus of each protein-coding gene in *S. cerevisiae* through homologous recombination [72]. When expressed, each recombinant gene fusion produced a C-terminally GFP-tagged protein. Notably this method did not alter the transcriptional regulation of genes; GFP-tagged remained under the control of their native promotes, reducing the odds of expression based artifacts. They screened the library fluorescence microscopy imaging tagged strains in log phase growth and classified the location of GFP-tagged proteins for thousands of strains across 22 subcellular localization categories, achieving ~ 75% coverage of the yeast proteome. Of particular importance to the work here, they classified some 800 proteins as cytoplasmic. Which means a protein was distributed randomly throughout the cytoplasm in their screen.

Cell-chip screen of stationary phase yeast GFP-library strains

Most microorganisms respond to starvation by arresting the cell cycle and enter a stationary phase, or G_0 state. In a typical yeast culture the transition to stationary phase begins following the exhaustion of glucose [101]. Stationary phase cells differ from proliferating cells in a number of cellular physiological and metabolic processes. Budding ceases, translation rates drop 300-fold, the cell wall thickens, trehalose accumulates as glycogen is exhausted [82]. Global RNA expression profiling, shows an upregulation of environmental stress response pathways, with a surprising similarity to the expression profile observed in heat shocked cells [102]. Interestingly, passage through stationary phase ages cells, as measured by reduced replicative capacity upon resuming proliferative growth [103], suggesting an accumulation of damage inherent in the transition.

Foundational experiments

As stated, my thesis work is based directly on the finding of widespread foci formation of GFP-tagged cytoplasmic proteins in stationary phase yeast cells as described by Narayanaswami et al. 2009. In short, the authors grew yeast GFP strains originally characterized as cytoplasmically localized in YPD for 60h to reach stationary phase and assayed the intracellular distribution of the tagged proteins using the automated cell chip microscopy platform. The authors found potential localization changes in 256 proteins, which were manually rescreened under the same conditions. The manual rescreening verified 180 proteins showed a strong change in distribution from diffuse to foci or fiber morphology. The dynamics of foci formation were characterized in detail to exemplify

the underlying diversity in regulation, and perhaps structure of morphological identical bodies. The two selected proteins were glutamine synthetase, Gln1-GFP, and pyrophosphate amino transferase, Ade4. These two showed differing induction and disappearance by metabolite depletion, translation inhibition, and protease inhibition. These two proteins were also extensively screened for co-localization with known cellular structures, but no colocalization was found, suggesting they are indeed novel bodies. However we can not rule out that some of the formed by the 178 untested GFP-tagged proteins may yet represent re-localization to known intracellular bodies.

We verified the reorganization of proteins into foci was not an artifact of the GFP-tag by two methods. The first was differential partitioning of proteins between the soluble and the insoluble phase. We found that a strong correspondence between cytoplasmic proteins that transitioned and those that formed foci in stationary phase, suggesting the mass spec is an orthogonal method of measuring protein phase change by a systematic, untagged method. The second experiment was to track localization changes in stationary phase cells with an alternate tag, namely the tandem affinity purification (TAP) tag, and fluorescently labeled antibodies. We found that TAP-tagged proteins showed the same foci formation in response to stationary phase and specific nutrient depletion as the GFP-tagged versions. Other groups have since tested many of these bodies with other tags (e.g. HA-tagged fibers by Noree et al. [41]), and in all cases found the assemblies to be independent of the C-terminal tag, suggesting intracellular body formation is an inherent property of many cytoplasmic proteins, in their untagged, native state.

Additionally, the results of two other experiments in the paper are revisited below as their full implications were not discussed in the paper. The first was the result of an experiment refeeding stationary phase cells containing Gln1-GFP foci with fresh media plus cycloheximide to block translation. If Gln1-GFP foci were storage bodies we might have observed a decrease in foci fluorescence intensity and an increase in cytoplasmic fluorescence intensity as Gln1-GFP proteins resumed their random distribution, as was seen in a similar experimental test of actin bodies [86]. Instead, we saw no dispersal. So either dispersal requires protein synthesis or Gln1-GFP foci are degraded in a translation dependent process. In support the second hypothesis is that one of the main effects of cycloheximide treatment is ubiquitin depletion, without which proteasome mediated degradation ceases [104]. Together with the results detailed in Chapter Two, this result argues Gln1-GFP foci are protein aggregates.

The second is the result of an experiment in which log phase cells expressing Gln1-GFP were transferred to synthetic defined media lacking nitrogen. Nitrogen depletion strongly up-regulates Gln1 expression [105], and we saw a strong increase Gln1-GFP fluorescence indicating an increase in protein concentration. If Gln1-GFP foci are aggregates and protein aggregation were solely concentration dependent [106], then foci should have formed. Instead glucose depletion was necessary for Gln1-GFP foci formation, which suggests either a metabolite specific regulation or an energy component to *in vivo* protein aggregation – a topic we delve into further in Chapter Four.

Future Chapters

Chapter Two provides evidence that the majority of foci discovered by Rammohan Narayanaswamy's screen the GFP-library in stationary are not functional complexes, but dysfunctional protein aggregates. We also present a correlation between aging and increasing Gln1-GFP foci formation, to suggest aggregation may be a common feature of cytoplasmic proteins with implications for cellular senescence.

In Chapter Three I measure global changes in the solubility of untagged proteins with age, as a systematic and unbiased verification of widespread decline in protein solubility in yeast over time. Many of the proteins showing this trend show a similar trend in aging worms suggesting the phenomenon may a conserved feature of aging.

Chapter Four lays out additional evidence supporting widespread protein aggregation in large scale studies and how they provide support for an evolved balance between protein aggregation propensity and expression level. We extend this model to include the potential of cellular energy to shift this balance through mechanisms of proteostasis control.

Finally, in Chapter Five, I discuss the possibility of conservation of widespread protein aggregation as a senescence factor during normal aging, speculate on general models for how complex quaternary structures form in the first place, and why they may be inevitable and propose a research direction to explore this new aspect of an old field.

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Chapter 2: Widespread protein aggregation redefines cytoplasmic architecture in stress and aging

INTRODUCTION

A plethora of new cytoplasmic structures formed by large protein assemblies have recently been discovered in eukaryotic cells. In yeast alone, nearly 200 normally diffuse cytoplasmic proteins, including enzymes from nearly all main branches of metabolism, can be induced to form large intracellular bodies. These bodies appear within cells as foci or fibers by fluorescence microscopy, and form in response to specific nutrient depletions [1] or stresses [41][108]. The foci/fiber-forming tendencies for many of these proteins are in some cases conserved across large evolutionary distances. For instance, cytidine triphosphate synthase has been observed to form fibers in bacteria, yeast, fly, and human cells, though the function for the fiber form of the enzyme is not yet known in any of these organisms [41–43]. The cellular functions of these assemblies are thus an open question for the majority of these cases.

Where cytoplasmic protein bodies have been well-studied, three cellular roles are observed: enhanced metabolic processing, storage depots, or aggregation. Many central metabolic enzymes are known to form complexes, varying greatly in both size and diversity of composition. For example, the purinosome is thought to be composed of enzymes carrying out the ten steps of *de novo* purine biosynthesis, as well as cofactor-producing enzymes [54]. Many of these proteins were reported to form a micrometer-sized body to increase the flux through this pathway [54][56], although there is some

evidence that the foci in human cells may represent overexpression artifacts [66]. On the other end of the functional assembly spectrum are the fibers formed by acetyl-CoA carboxylase and beta-glucosidase, which are nearly homogeneous strands no more than 20 nm wide. Both naturally occurring and artificially scaffolded metabolic pathways show improved flux [109] [110]. The evolutionary conservation of many of these structures, such as CTP synthase mentioned above, is often held up as evidence for a retained, undiscovered function. An alternate hypothesis is that the enzymatic structure optimal for catalysis is prone to nonfunctional or even dysfunctional oligomerization, and the conservation of function inherently preserves fiber formation [Ann Rev]. Additionally, some enzymes assemble into foci under conditions when their pathway's end product is limiting and requires synthesis, such as adenine for Ade4p or pyruvate and ATP for Tdh1p [1].

Alternatively, there are many proteins that assemble into storage bodies. Under cellular stress or yeast quiescence, filamentous actin dissembles its web-like network and contracts into discrete structures called actin bodies. Upon the resupply of nutrients, the actin bodies dissolve and actin returns to its normal fibrous form [86]. Similar conditions induce the formation of p-bodies and stress granules for the storage of mRNA along with a host of mRNA-binding proteins and components of the translational machinery [111][112][113]. In higher eukaryotes these mRNA bodies are formed by the aggregation of mRNA-binding proteins into unusual beta-amyloid aggregates, structures more typically associated with aggregation [114].

In yeast, there are a number of bodies where aggregated proteins are shunted. For refolding or degrading, proteins collect in the 'juxtanuclear quality control' compartment JUNQ [90], endoplasmic reticulum associated protein degradation (ERAD) machinery [90], or aggresome [89], all of which appear as paranuclear foci by fluorescence microscopy. Permanently misfolded proteins are sequestered near cells' exterior in distinct foci dubbed 'insoluble protein deposits' (IPODs) [90]. Mounting evidence suggests that many types of aggregation are highly specific for similar sequences. Thus, even within the above mentioned bodies, particular aggregated proteins may be associated largely with themselves and chaperones. Yeast foci often appear during the nutrient depletion phase of the post diauxic shift, which is the transition to stationary-phase culture conditions and is stressful due to nutrient depletion and the accumulation of toxic metabolites [82]. Formation in response to stressful conditions is a hallmark of aggregation, supporting the hypothesis that many of these newly discovered protein assemblies are stress bodies.

Here, we systematically screened the yeast proteome under various stresses to detect foci-forming proteins and to determine which of the three above-mentioned hypotheses best accounts for the role of these novel bodies.

RESULTS AND DISCUSSION

Testing for multienzyme assemblies of pathways

Many of the proteins that were previously observed to form foci in a non-directed screen were also found to participate in known metabolic complexes in yeast or other organisms. Amongst the complexes associated with foci were the trehalose synthase complex and the tRNA multisynthetase complex [115], [116]. There was also significant enrichment among foci-forming proteins for nitrogen metabolism and purine biosynthesis (as quantified by Gene Ontology annotations, Table S1) and for the previously observed purinosome.

Based on these observations, we hypothesized that foci are composed of enzymes within a given pathway, potentially forming functional factories that improve metabolic efficiency. However, if this were the case then it might also be expected that co-localized proteins would also show similar kinetics of foci formation. Therefore, as a simple test of pathway assembly, we measured whether members of metabolic pathways show similar patterns of induction of foci formation in response to specific or general nutrient depletion (Figure1A). We assayed foci formation by 75 GFP-tagged proteins from 8 major cytoplasmic processes selected from the Gene Ontology annotations enriched in our previous screen of all cytoplasmic proteins [1] and pathways upregulated in stationary phase [102]. GFP-tagged strains were picked from the GFP-strain collection [117] and grown in each of 5 metabolically-limiting conditions. Fluorescence microscopy images were taken and scored for the presence or absence of foci as a binary score for a

total of 370 fluorescence microscopy screens. We compiled these binary scores into a dissimilarity matrix and applied unsupervised hierarchical clustering using correlation distance at the measure. Surprisingly, hierarchical clustering did not group together members of a given cellular process which indicates there was little correlation in their foci induction patterns.

Among the most correlated sets, as measured by co-clustering, were proteins from *de novo* purine biosynthesis and chaperone processes. This observation nominally seemed to support the conservation of the hypothesized purinosome complex between yeast and human. However, only Ade4p formed foci in response to end-product depletion (Figure 1B), a striking contrast to what has previously been observed in humans. In transiently transfected human cells, fluorescently tagged versions of all 6 enzymes of the *de novo* purine biosynthesis pathway formed foci when grown in media dialyzed to remove purines [56]. Furthermore, the assemblies formed by various human enzymes of the *de novo* purine biosynthesis pathway co-localized with each other in transfected cells expressing pair-wise combinations of the fluorescently-tagged pathway members. We conducted a similar test for colocalization in yeast under general nutrient depletion by transfecting GFP-tagged strains with plasmid expressing Ade4-mCherry. Of all the pairs tested, Ade4-mCherry only co-localized with Ade4-GFP (Figure 1C), again contrasting the results seen in humans as evidence for a hypothesized purinosome.

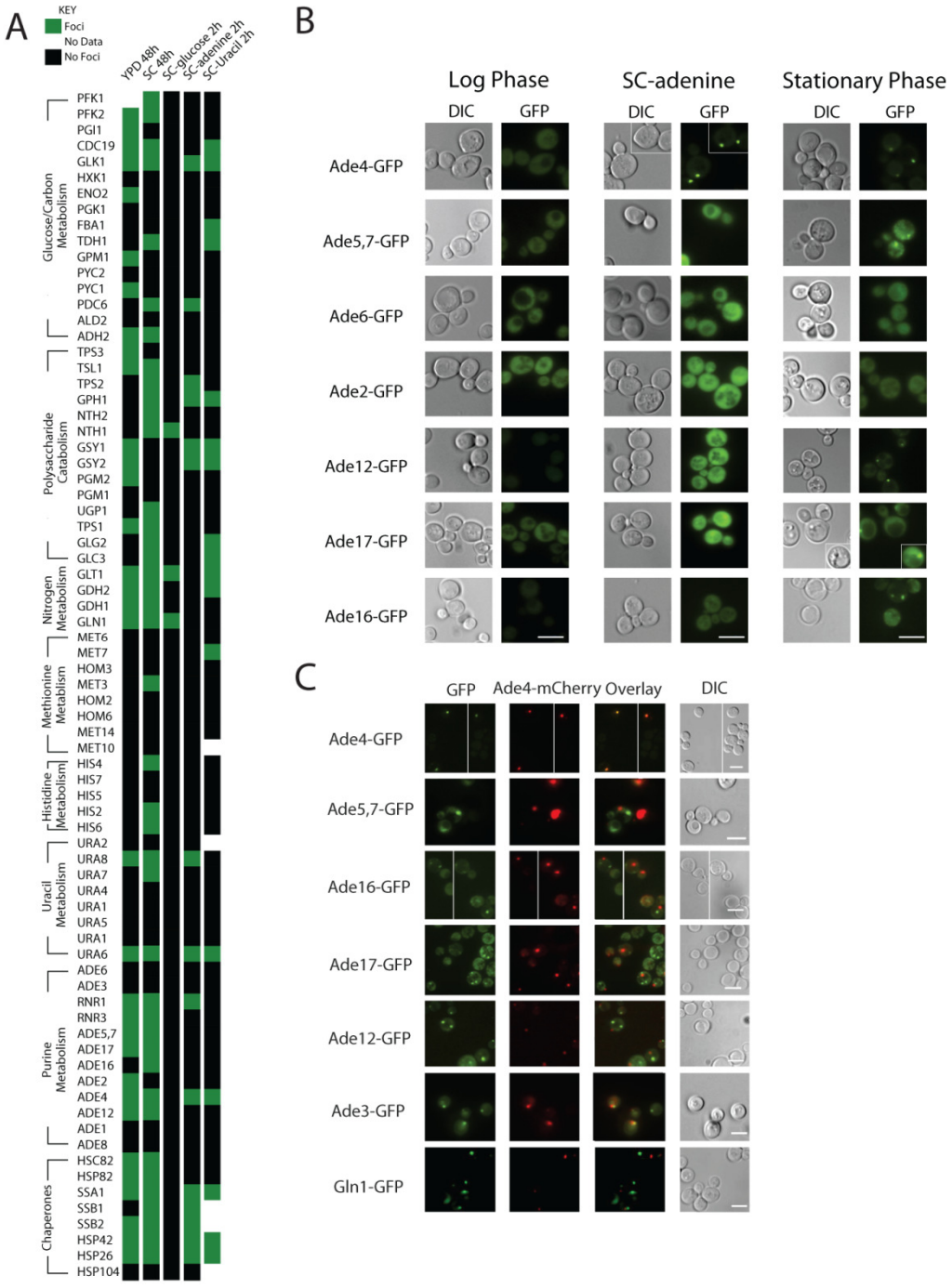

Figure 2-1. Disparate induction patterns argue that metabolic enzymes in the same pathway do not form foci in a coordinated fashion.

A) A test of 75 GFP tagged proteins spanning 8 major cellular processes for their tendency to form in each of 5 metabolically limiting conditions found little concerted foci

induction B) The de novo purine biosysnthesis pathway illustrates to case; only the enzyme responsible for the first step, Ade4-GFP, forms foci in response to adenine depletion. C) Even under conditions where other members of the purine biosynthesis pathway formed foci, Ade4 did not colocalize with any other pathway members. The absence of coordinated induction, a hallmark of co-complex members, argues that most foci do not represent pathway specific multi-enzyme complexes.

Heat shock causes similar sets of cytoplasmic proteins to become insoluble and form foci

We next tested the possibility that the formation of foci was the result of a general collapse of proteostasis, leading to aggregation of normally soluble, diffuse proteins. If foci form by aggregation, then other stresses that lead to protein aggregation should induce foci formation as well. Heat shock, for instance, causes protein unfolding and triggers a number of response pathways to prevent, sequester, or degrade aggregated proteins. We systematically screened for changes in protein aggregation in response to heat shock by measuring the relative solubility of untagged cytoplasmic proteins in whole cell lysate by gross fractionation shotgun proteomics (Figure 2A). Mass spectrometry analysis of whole cell lysate was used to measure the solubility of roughly half of all cytoplasmic proteins (395 out of ~800).

By comparing the relative solubility changes of cytoplasmic proteins between heat-shocked and normal log-phase cells, we explicitly controlled for any possible bias introduced by lysis that might induce insoluble partitioning. Following heat shock, 117 of these proteins became significantly more insoluble (Z-score $\geq$ 1.96, Table S2) relative to the control. This set overlaps significantly with the set of proteins that formed foci in

stationary phase [1] (Figure 2B), suggesting that a subset of foci formed at stationary phase may also be formed by heat shock.

Increased insolubility was previously observed to be strongly correlated with foci formation [1]. To test if increased insolubility due to heat shock was due to aggregation of proteins into foci, we repeated the heat shock experiment with GFP-tagged strains for four proteins from the intersection: Gln1-GFP, Gpm1-GFP, Ssa1-GFP, Ura7-GFP (the more highly expressed paralog of Ura8), and two proteins from outside the intersection (Yef3-GFP and Rpl24a-GFP). The four proteins that formed foci in stationary phase also formed foci following heat shock (Figure 2C), whereas the two proteins from outside the intersection did not form foci (data not shown). Interestingly, there was a difference in the population penetrance of the foci phenotype following heat shock compared to stationary phase, with fewer cells showing single, distinct foci and more cells showing a number of small foci, marked as indeterminate (Figure 2D). This difference is particularly stark for Gln1-GFP, where 90% of cells have a single clear protein aggregate in stationary phase compared to 25% in heat shock. This difference may reflect an impairment of cells' ability to traffic small aggregates into a single large aggregate.

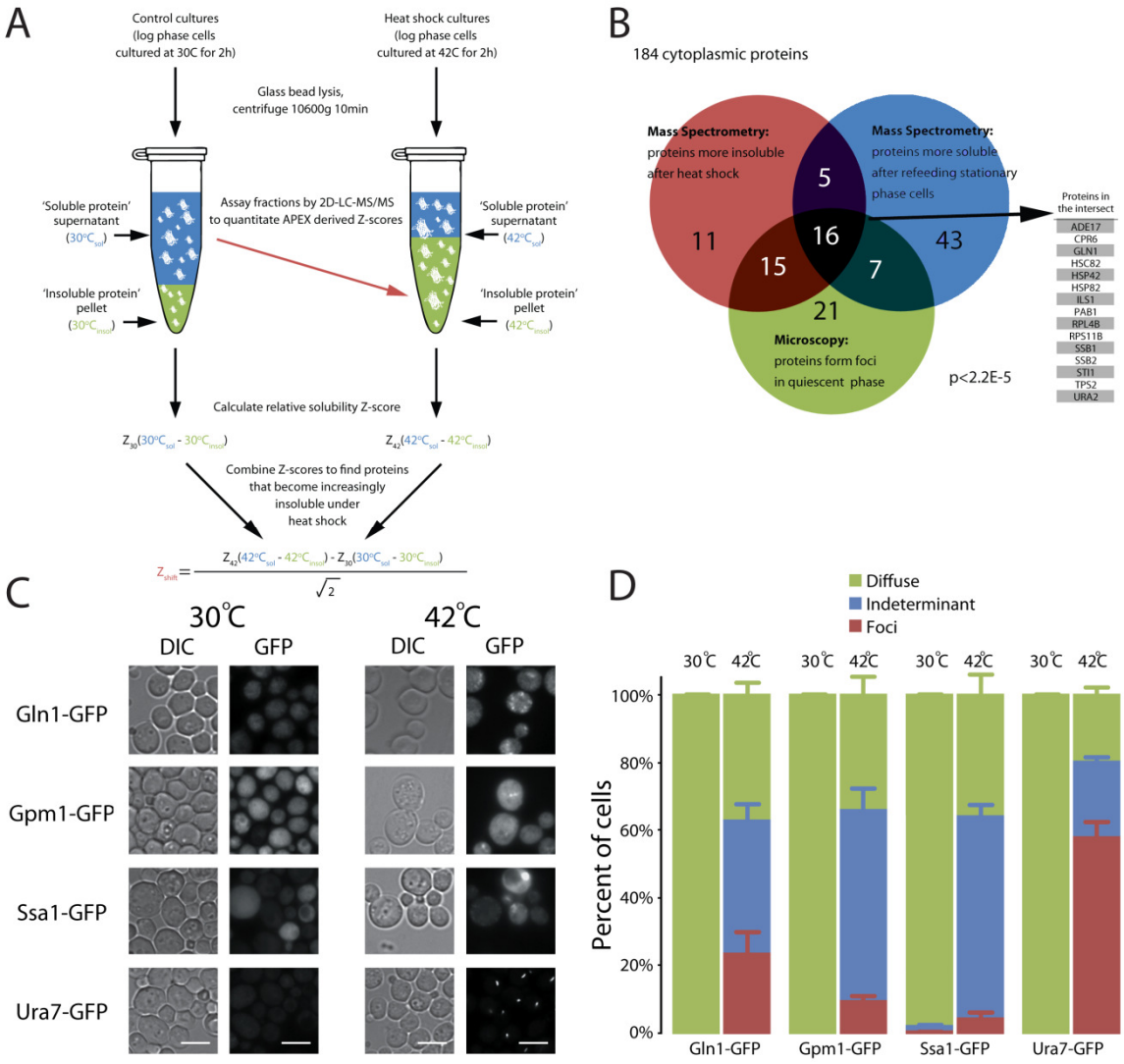

Figure 2-2. Foci forming proteins are significantly more likely to precipitated *in vivo* following heat shock suggesting they are assembling into stress bodies.
A) Experimental design for measuring solubility changes of untagged proteins during heat shock as the relative partitioning between the soluble and insoluble phases compared to controls. B) The set of proteins that become insoluble during heat shock significantly overlapped those that formed foci when GFP-tagged and those detected in our previous stationary phase mass spectrometry assay. Probability of overlaps was calculated as the iterated cumulative hypergeometric probability of all three sets. Proteins seen in all three sets are shown in the inset table. C) Proteins from the intersect of the GFP-tagged protein screen and those insoluble following heat shock were tested and found to form foci in response to heat shock. D) Quantifying change in GFP-tagged protein distribution shows a specific and distinct shift toward foci formation following heat shock.

Co-immunoprecipitation of foci forming proteins

What then are foci composed of? We took a targeted approach to characterizing the components of a subset of foci by immunoprecipitating the protein assemblies and identifying the co-immunoprecipitating proteins. We began by purifying foci formed by the glutamine synthetase, Gln1-GFP. These Gln1-GFP foci survive cell lysis ostensibly intact, even apart from cellular debris (Figure 3A), and are strongly and specifically purified by antibodies targeting the GFP fusion (Figure 3B). We found that Gln1-GFP foci are composed almost exclusively of Gln1-GFP and the ubiquitous hsp90 chaperones Hsp82p and Hsc82p (Figure 3C, Spreadsheet S2 for full results). This differs markedly from co-purifying partners seen in log-phase cells in a TAP-tag screen, where Gln1 was associated with trehalose synthase (Tps1p) and the small heat shock protein Hsp42p. We constructed a dually fluorescent protein-tagged yeast strain in order to confirm the association and found that Gln1-GFP foci co-localized with Hsc82-TagRFP foci (data not shown).

Hsp82-GFP

While Hsp82-GFP foci do not detectably persist through cell lysis, those interactions that do persist should be a subset of the original composition. Among the set co-immuno-precipitating proteins a significant number of the cytoplasmic proteins also form foci in GFP tagged strains ($p<1.0E-3$, Table S3), including a reciprocal enrichment for Gln1p. This suggests a role for the cytoplasmic Hsp82 in chaperoning a subset of foci-forming

proteins in subcellular structures, reminiscent of similar findings for IPOD and JUNQ forming proteins [90].

Ils1-GFP

We see a different chaperone association for isoleucine tRNA synthetase, ILS1-GFP, which elutes primarily with the ribosome associated hsp70 chaperones, Ssb1p and Ssb2p (Figure 2C). The other members of the ribosome-associated complex (RAC), Ssz1p and Zuo1p, also co-purified (Spreadsheet S1). The RAC chaperones nascent polypeptide chains during translation. The tRNA synthetases for methionine, alanine, valine, and threonine as well as the tRNA structural protein, Arc1p co-purified with ILS1-GFP. Of these, all but Mes1-GFP formed foci in stationary phase cells. Curiously we also see enrichment for several paralogs of the main structural protein of virus-like particles. This differs from the results of log phase protein interaction studies where no chaperone interactions have been reported.

Cdc19-GFP

Cdc19-GFP also shows enrichment for hsp70 class chaperones: Ssz1, Ssa2, and Ssb2 (Figure 3G). Also detected, where Hsp82 and Hsp104 though with less significant Z-scores and fold enrichments. More significantly enrichments were measured for several translation associated proteins, as well as fellow members of glycolysis, most strongly for Pdh1p. Among these is Acs1 whose paralog, Acs2, was detected as an interaction partner in log phase protein interaction screen [118].

Ade4-GFP

The first step in the de novo purine biosynthesis pathway is catalyzed by Ade4p. In stationary phase cells Ade4-GFP co-purifies most strongly with a number of Golgi proteins, notably the alpha-1,6 mannosyltransferase complex, (Hoc1p, Anp1p, Mnn9p, Mnn10p, and Mnn11p)(Figure 3H). There was also enrichment for Udf1p and Cdc48p which deliver poly-ubiquinated proteins to the proteasome for degradation. A weak, but significant enrichment was measured for only one other member of the de novo purine biosynthesis pathway, namely Ade5,7p. Perhaps the minor subset of diffusely distributed Ade4p possess such interactions, however no colocalization was detected between Ade-mCherry foci and Ade5,7-GFP foci (Figure 1C).

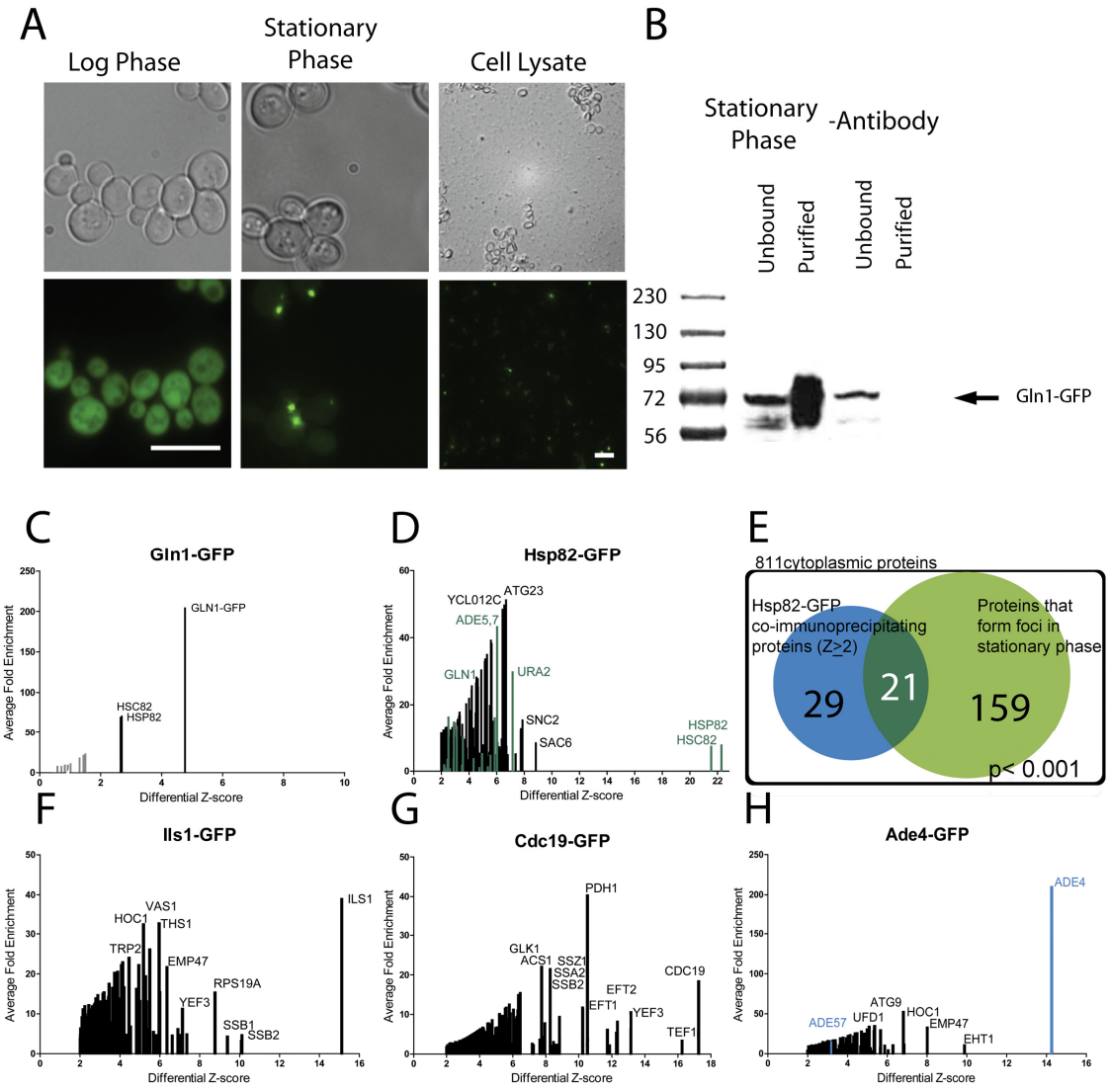

Figure 2-3. Immunopurification reveals diversity of foci composition, with a common theme of protein quality control.

A) Gln1-GFP, while diffuse in log phase cells, localized predominantly into foci in stationary phase cells-foci which persisted in lysate and B) could be selectively purified by immunoprecipitation with goat anti-GFP antibodies and visualized by western blot with mouse anti-GFP antibodies. Proteins that coimmunopurified with various GFP-tagged, foci-forming proteins were assayed by shotgun mass spectrometry. Identified proteins are arranged by significance of enrichment relative to the untagged control strain, BY4741, on the X-axis and the fold change of enrichment on the Y-axis for each bait protein tested. C) Immunoprecipitation of Gln1-GFP from stationary phase cells co-immunoprecipitated the cytoplasmic hsp90 class heat shock proteins Hsp82 and Hsc82. D) Conversely, among the many interaction partners identified with Hsp82-GFP, Gln1

was reciprocally enriched. E) Of the 50 cytoplasmic proteins that copurify with Hsp82-GFP a significant number (21, shown in blue in D) have been observed to form foci ($p<0.001$, Table S3) suggesting a subset of foci forming proteins are hsp90 clients in their foci forming state. F) Ils1-GFP also copurified with chaperones, but in with the hsp70 class chaperones Ssb1 and Ssb2. There were also several other tRNA synthetases that co-purified. G) Cdc19-GFP also copurified with hsp70 class chaperones as fellow members of glycolysis, notably Pdh1p. H) Ade4-GFP showed no significant chaperone interactions; instead interactions with Golgi to ER transport proteins were enriched, as well as autophagy proteins, Atg9p, Atg23 and Vac8, as well as ERAD components, UFD1 and CDC48.

Arsenic induced insolubility recapitulates stationary phase induced insolubility

Arsenic is thought to be toxic to cells both by damaging cytoplasmic proteins directly by reacting with reduced thiol groups [119] destabilizing and unfolding them, and by inhibiting chaperones that would help fold unfolded nascent chains and misfolding proteins [120]. Jackobson *et al.* reported widespread protein aggregation in yeast after treating cells with 1.5mM arsenite, As(III), for one hour as measured by shotgun mass spectrometry of fractionated cell lysate. Overall, 143 proteins showed a significant shift toward the insoluble fraction following arsenic treatment, 59 of which were cytoplasmic proteins (Table S4). Comparing this set of 59 proteins to the set of 114 proteins that were more insoluble in stationary phase[1], we find a significant overlap of 22 proteins (Hypergeometric test, $p < 2.8E\text{-}6$, Figure 4A). Both conditions also induce foci formation. For instance the AAA chaperone Hsp104-GFP and stress granule marker Pab1-GFP both form foci in each condition (Figure 4B) [1] [120]. Similar to the heat shock experiment, these results suggests a many cytoplasmic proteins aggregate in response to diverse types of stress, and a common subset of chaperones respond to sequester the aggregates, including forming foci.

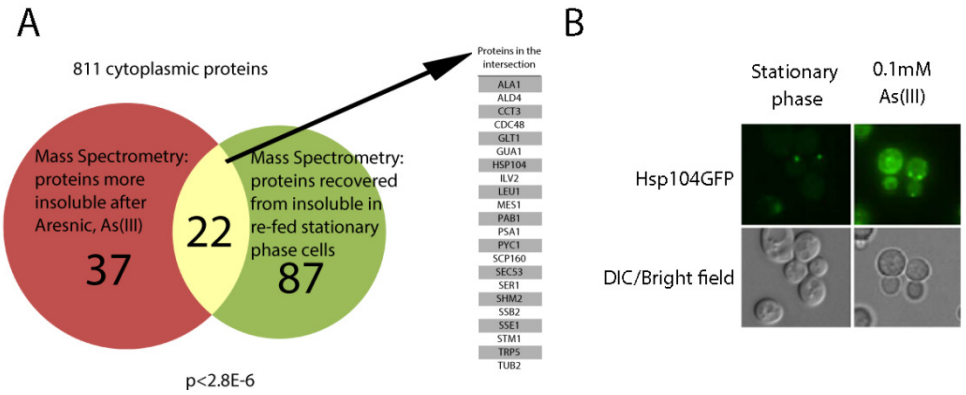

Figure 2-4. A similar set of cytoplasmic proteins aggregate and form foci in response to arsenic and stationary phase nutrient depletion.
A) A Venn diagram illustrating the statistically significant overlap between proteins that become more insoluble following arsenic treatment of yeast cells and proteins more insoluble in stationary phase cells. B) Log phase cells stressed with 0.1mM arsenic[120] and stationary phase cells both harbor aggregate bodies, which appear as foci in GFP-tagged strains, such as Hsp104-GFP shown here.

Gln1-GFP foci formation correlates with aging

Many mechanisms cells use to handle and clear protein aggregates decline with age across model systems. Chaperone expression decreases [121], proteasome activity diminishes [122], and chaperone mediated autophagy slows with increasing cellular and organismal age [123]. This potentially contributes to the accumulation of aggregation in several age related diseases, such as plaques in Alzheimer's disease [124], and aggregates of any other protein as well. Recent work in worms suggests widespread aggregation occurs normally in the course of aging [125]. If the foci are the result of general aggregation, we speculated their incidence should be correlated with increasing cell age. Two easily noticeable phenotypes for aging in yeast are expansion of cell volume [126], and the accumulation of bud scars [127],[128]. We used Gln1-GFP foci formation as a

model foci forming protein due to its high signal to noise ratio. Cell size and foci per cell, as measured by fluorescence microscopy, revealed a correlation between the frequency of Gln1-GFP foci formation and cell size (Figure 5A R^2=0.78). Repeating the experiment by, fluorescence activated cell sorting (FACS) of cells by size again found cells with foci were significantly larger than cells without foci (Figure 5B, $p< 0.0001$). Similarly, old cells were enriched by FAC sorting cells by fluorescence intensity for bud scar marker, Calcofluor white. Cells with more bud scars were more likely to contain more foci, though the correlation was not linear, with a large jump in the mean number of bud scars between cells with no bud scars and cells with 1 bud scar (Figure 5C). The difference was even more striking when we narrow the analysis strictly to pairs of mother and daughter cells. Mother cells were significantly more likely to have Gln1-GFP foci than the daughter cells (Figure 5D,t-test $p< 3.2\text{-}10$) suggesting retention of foci by mother cells, a hallmark of aging factors in yeast.

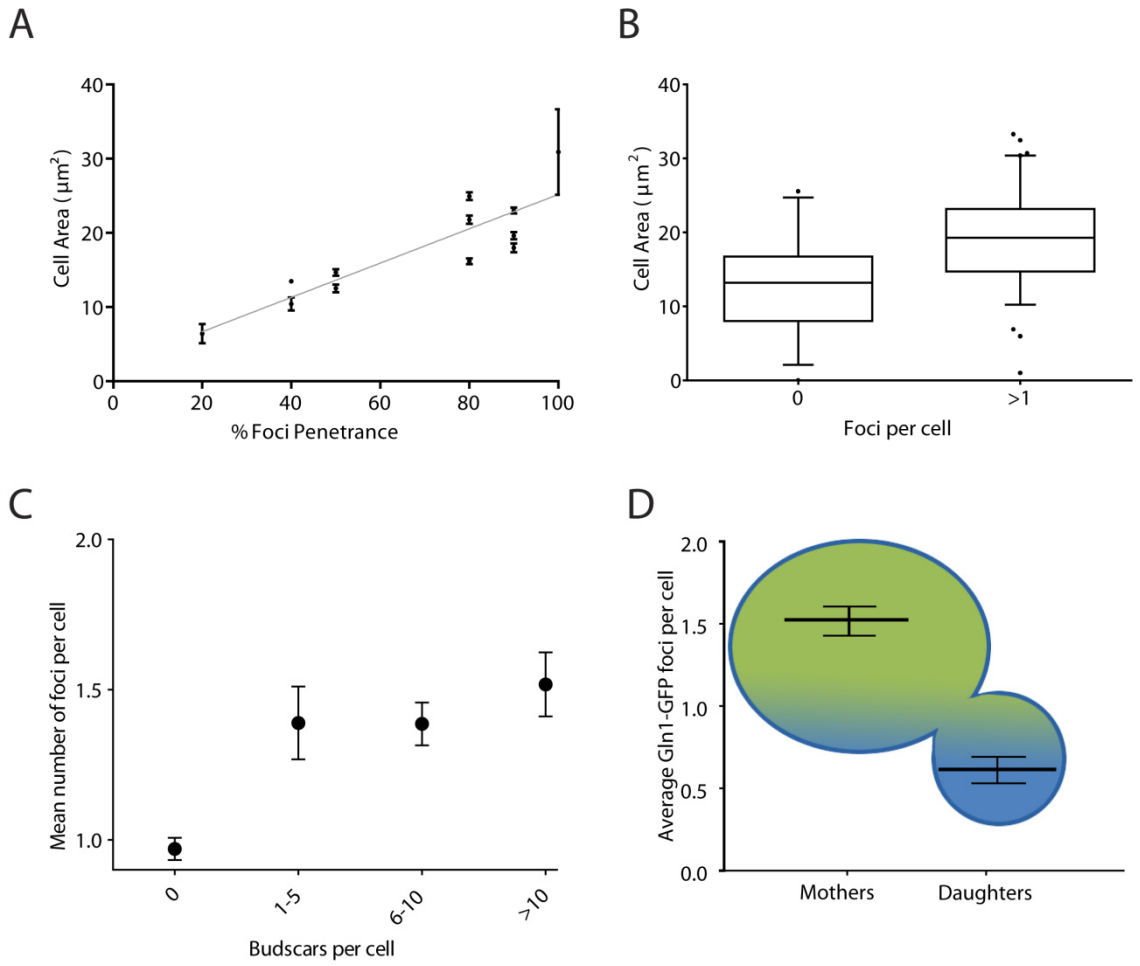

Figure 2-5. Gln1-GFP foci formation correlates with markers of cellular age.
A) Increasing cell size is a marker for age, and positively correlates with likelihood to show Gln1-GFP foci as measured by a manual screen (R2=0.78). B) Gln1-GFP foci formation is more likely in larger cells as assayed by Fluorescence Activated Cell Sorting (FACS) cells by size and manually scoring foci. C) Similarly, FAC sorting cells by bud scar and manually counting foci per cell found cells with more bud scars were more likely to contain more foci. D) Reproductively older mother cells, as measured by bud-scars, are more likely to have Gln1-GFP foci and multiple foci than reproductively naive daughters.

Rapamycin treatment suppresses Gln1-GFP foci formation

Rapamycin is one of the few chemical interventions that increases lifespan of cells in culture across a wide range of eukaryotes, including yeast [129], worm [130], fly [131], mouse [132], and human [133]. Within cells, rapamycin binds to FK-binding protein 12, which then binds to and inhibits the highly conserved TOR Compex1 (TORC1)[134]. TORC1 activates metabolism to fuel the cell cycle in proportion to available metabolites. When TORC1 is inhibited cells behave as if starved, arresting division in G_0 [135] and releasing the repression of autophagy and the Msn2/Msn4-mediated stress response pathways [136][137]. Rapamycin also decreases translation initiation and ribosome biogenesis, collectively decreasing the steady-state protein concentration inside cells [138]. Together, these effects reduce the protein aggregation load within a cell. Recently, rapamycin derivatives have even shown promise in treating diseases caused by aggregation, including progeria [133].

Because foci formation increases with aging, we hypothesized that rapamycin treatment, which decelerates aging and activates stress response pathways, should prevent foci formation. To test this hypothesis, we grew Gln1-GFP tagged yeast in increasing concentrations of the drug for 24 hours. Using imaging flow cytometry, we developed a protocol to automatically image and score foci within tens of thousands of cells per sample, in this manner achieving more accurate and unbiased statistics (Figure 6A). We found a clear dose-dependent reduction in Gln1-GFP foci in response to rapamycin

treatment (Figure 6B). Together, these results show that Gln1-GFP foci bear many of the traits of age-dependent aggregation.

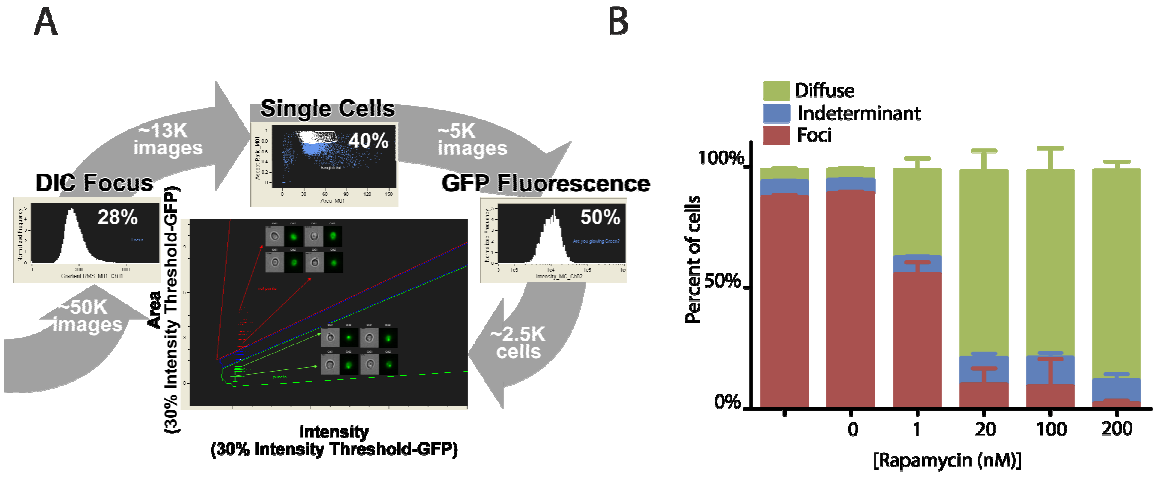

Figure 2-6. Rapamycin treatment prevents Gln1-GFP foci formation.
A) The proportions of cells exhibiting visible Gln1-GFP foci were measured in an unbiased fashion by automatically imaging cells at high-throughput using imaging flow cytometry (ImageStream), and identifying and quantifying the relevant cell subpopulations as shown. B) Using this strategy, we observed that rapamycin treatment induced a dose-dependent reduction in the proportions of cells with Gln1-GFP foci formation across a concentration range of 1 to 200 nM.

Most foci are not multi-enzyme factories

Massive rearrangements of cytoplasmic structures occur in response to a variety of stresses. Notably, many proteins condense into concentrated pockets [139][113][1], effectively decreasing the concentration of normally diffuse proteins. These changes alter translation, chaperone capacity, and likely metabolism in the cytoplasm, both within and outside the foci. There are many examples of metabolically active, heterogeneous protein megacomplexes whose formation is regulated in response to nutrient availability. Notable examples include carboxysomes [140], cellulosomes [141], and perhaps

purinosomes in humans [67]. Most of the observed yeast protein foci are inconsistent with such structures; we observed that proteins within the same pathway do not generally form foci under the same induction conditions. If proteins do not form assemblies at the same time, it precludes the possibility of their co-assembly into the same structures. In particular we concluded that purine biosynthesis enzymes in yeast do not collectively form foci representing purinosomes. More generally, foci do not appear consistent with multi-enzyme factories catalyzing consecutive metabolic reactions.

The two classes of proteins that showed the greatest coordinated induction in this study were chaperones and *de novo* purine biosynthesis enzymes. For the latter, we did not observe colocalization of the enzyme carrying out the first step of the pathway, Ade4p, with other enzymes in the pathway (and, as noted previously, observation of colocalization in human cells depend strongly on overexpression [66], raising the possibility that they represent aggregates). Similarly, pull-downs of Ade4-GFP immunoprecipitated one other members of the purine biosynthesis pathway, Ade5,7, and the association was weaker (<1/20 of the abundance of Ade4-GFP) than other presumably non-specifically associating proteins. Such weak associations may either represent a highly unstable complex, or more likely—given the lack of fluorescence co-localization—associations with the minor population of diffuse Ade4-GFP rather than the major population of foci-associated Ade4-GFP. Studies of physical associations in log-phase cells have not found any association between the members of the purine biosynthesis pathway and Ade4p, other than oligomerization of Ade4p itself. Rather than

forming a purinosome, the data may suggest that Ade4p either forms large homo-oligomers, as for acetyl-CoA carboxylase, or an aggregate. Thus, in general, foci do not appear to be consistent with the organization of more extensive metabolic pathways for the purposes of substrate channeling or enhanced metabolic efficiency.

Many foci are novel stress bodies

While proteins, particularly RNA binding proteins and chaperones, are known to form stress bodies in response to nutrient depletion or heat shock, the scope of additional proteins from diverse pathways added to the list in this study is unprecedented. Having ruled out the hypothesis that the foci we see represent a vast array of pathway spanning multi-enzyme complexes, the next logical hypothesis the data support is novel stress bodies. Many proteins from central metabolism, translation, and chaperones collapsed into insoluble bodies in response to a variety of stresses. These bodies could either be storage bodies, like actin bodies, or general aggregation like aggresomes. However, the line between these alternatives blurred in the wake of recent work showing RNA granules are formed by β-amyloid aggregation of low complexity regions within partially unfolded RNA-binding proteins[114]. The granules appear as foci within cells and are insoluble upon lysis. However, the proteins retain their RNA-binding capacity, and unlike amyloid aggregates associated with prions or neural plaques, are highly dynamic and specific only the type rather than the sequence of amino acid side chains.

General, widespread aggregation of cytoplasmic proteins

There are several lines of evidence that many of the foci are aggregates. We previously found foci forming proteins were computationally predicted to have a higher propensity to aggregate than non-foci forming proteins using the TANGO algorithm[142]. Stressing cells with heat shock or arsenic, which are known to cause aggregation, drive to the insoluble phase many of the same proteins that form foci. Indeed insoluble shifts were predictive of foci formation. Of the foci forming proteins we studied in detail, Gln1 seems the most likely to be forming aggregates; it forms foci in response to nearly every stress tested, co-immunoprecipitates with chaperones, and its percent penetrance in the population correlates increasing aging phenotypes.

Further, Gln1-GFP foci are unlikely to be a storage body as foci formed by nutrient depletion did not disperse when new media with cycloheximide was added [1]. So recovery of diffuse Gln1-GFP requires new protein synthesis. One of the main effects of cycloheximide treatment is the depletion of ubiquitin [104]; so perhaps the required protein synthesis required for Gln1-GFP foci disappearance is additional ubiquitin to target it to the protein quality control machinery. We speculate that the set of proteins that, like Gln1, shifted to the insoluble phase in response various stresses are forming foci composed of aggregates. Top candidates would be foci forming proteins that show novel chaperone associations in stationary phase or under stress, such as the foci forming proteins that co-immunoprecipitated with Hsp82-GFP.

Given the fitness cost of clearing aggregates formed by the expression of one a non-functional gene is above 3.2% decrease in growth rate [143], why do cells allow up to 20% of cytoplasmic proteins to crash out at a common, even routine stress such as nutrient depletion? One possibility is many evolutionarily ancient proteins are trapped in a local minimum in sequence space with limited options to explore while maintaining essential functions proteins. These proteins may be expressed near the limits of solubility or beyond in order to cope with the stress and cells accept the costs of cleaning up aggregated proteins to achieve higher enzyme concentrations. A second alternative is aggregation serves a function for rapidly changing protein activity, e.g. RNA granule formation mentioned above.

CONCLUSION

In summary, our findings suggest widespread foci formation represents extensive protein aggregation that dramatically alters the organization of the yeast cytoplasm under stress. Our results imply many of the assumptions about cytoplasmic enzyme availability - based on a random distribution in the aqueous phase - are not true in stressed and stationary phase yeast. Investing whether a similar phase change restructures cytoplasmic protein architecture inside organisms in quiescence and stress would be a fascinating basic research project with obvious health implications if found in human cells.
Future work to test the generality of the storage body hypothesis would be useful to concisely establish if any foci are storage bodies. One possible experiment to accomplish this would be a mass spectrometry assay of pulse-chase labeled proteins partitioning between soluble and insoluble phases in starved compared to re-fed cells.

METHODS

Media and yeast strains

Yeast strains had a genetic background of BY4741 [genotype, MATa his3_1 leu2_0 met15_0 ura3_0]. Strains expressing GFP-tagged proteins were obtained from the OpenBiosystems GFP collection. Rich (YPD) medium containing Yeast Extract (1%), Peptone (2%) and glucose (2%) was purchased from Sunrise Sciences. Synthetic complete medium (SC) contained 1x Yeast Nitrogen Base (BD Biosciences/Difco) without amino acids, synthetic drop-out medium supplement mix (Sigma) with (or without) glucose (2%) or was purchased premixed from Sunrise Biosciences. Cultures were started from by picking from freezer stocks into YPD and growing overnight before transferring to new media for regrowth. All cultures were maintained shaking at 30°C unless otherwise specified.

Foci induction by metabolite depletion

Select GFP-tagged strains were picked into a96-well plate and grown overnight in YPD. Cultures from this plate were divided into a dozen plates for storage single use glycerol stocks. For each experiment a plate was thawed and used to inoculate a YPD plate for overnight growth. Overnight cultures were used to inoculate a new plate to 0.2 OD/ml in YPD or SC for nutrient dropout and SC growth to stationary phase. For media exchange experiments, cells were regrown to approximately 1 OD/ml in SC, washed once with the destination media, and then resuspended in SC minus the specified metabolite for two hours to induce foci. Cells were fixed with 4% formaldehyde (SPI-CHEM) at

room temperature for 60 min, and washed with PBS before storing in PBS at 4C until imaging.

Cells were imaged on either a Nikon E800 fluorescence microscope with Photometrix Coolsnap CCD camera under oil immersion at 100x magnification or on a Nikon TE2000-E with a Photometrics Cascade II camera under oil immersion at 60x magnification. Differential interference contrast and widefield fluorescent images were collected using standard filter sets and processed using Nikon Elements AR.

Colocalization experiments

Strains for testing colocalization were created by transforming strains from the GFP collection with a high copy plasmid (pRS426) expressing Ade4-mCherry under the control of the GPD promoter. Strains were grown 48 hours in SC-uracil to stationary phase to induce foci, fixed and imaged as above.

Immunoprecipitation of GFP-tagged proteins

Strains from the GFP collection or the parental strain BY4741 were cultured in SC medium to stationary phase (48 hours) at 30˚C to reach 100OD of cells per sample. Foci formation was verified by microscopy before lysing cells by vortexing with glass beads in 50mM Tris, 50mM NaCl, 5mM $MgCl_2$, 1X Protease Inhibitor Cocktail (CalBiosciences), and 1mM DTT. After verifying by microscopy that foci were retained in the lysate, GFP-tagged proteins were immunoprecipitated with 4µg rabbit anti-GFP antibodies (Sigma) bound to 200µl Protein A-conjugated Dynabeads

(Immunoprecipitation Kit, Invitrogen) according to the manufacturer's instructions. Immuno-precipitated proteins were washed three times with PBS (pH 7.4), and eluted by incubating beads in 120µl 50% trifluoroethanol at 70°C for 10 minutes. For Western blot verification, samples were eluted by boiling protein-A beads in SDS-PAGE loading buffer for 5 minutes and detected with mouse anti-GFP primary antibodies (Covance) and horseradish-peroxidase conjugated goat anti-mouse secondary antibodies (Santa Cruz Biotechnology), visualized by luminol (Santa Cruz Biotechnology).

To measure the relative enrichment of proteins between the GFP-tagged strains and the untagged parental strain, we compared spectral counts of a given protein between samples [144] as described below. Proteins with $Z \geq 2.0$ were significantly enriched (97.7% confidence level) by immunoprecipitation.

Heat shock experiments

For heat shock experiments, cultures were restarted in SC to 0.2 OD/ml in triplicate for each strain, regrown to approximately 1 OD/ml, and transferred to shaking incubators at either 30 °C or 42 °C for two hours. For mass spectrometry, cells were lysed as described above and fractionated to recover a soluble and an insoluble faction for each sample as previously described [1]. To measure foci induction, cells from the various GFP strains were fixed and imaged as described above.

Mass spectrometry

Proteins from whole cell lysates and immunoprecipitation were prepared for mass spectrometry denaturing with 50% trifluoroethanol (TFE) (v/v). Proteins were reduced

with 15 mM DTT at 65°C for 45 min and alkylated with 55 mM iodoacetamide at for 30 min on the bench. Samples were diluted 10-fold with digestion buffer (50 mM Tris–HCl pH 7.8, 2 mM $CaCl_2$) and digested with with 2 µg Proteomics Grade trypsin (Sigma) during a 4h incubation at 37°C or overnight on the bench. Trypsin activity was halted by the addition of 1% formic acid, and sample volume was reduced to ~100 µl by SpeedVac centrifugation. Samples were resuspended to 150 µl with Buffer C (95% H_2O, 5% acetonitrile (ACN), 0.1% formic acid). To remove undesirable contaminants, digested samples were cleaned up by reverse phase chromatography on Hypersep C18 Columns (Thermo). Whole cell lysates were also filtered on Microcon 10 kDa Spin Tubes (Millipore).

LC/MS/MS analysis of whole cell lysate samples and Gln1-GFp IP samples was carried out on an Eksigent nanoflow LC system connected to an LTQ-Orbitrap XL (Thermo) as previously described [144]. Each biological replicate was sampled by four injections. LC/MS/MS analysis of the remaining IP samples were carried out on an Eksigent nanoflow LC system connected to an LTQ-Orbitrap Velos (Thermo).

Bioinformatic analysis

Spectra from the whole cell lysate were searched against the non-redundant yeast protein-coding data set downloaded from SGD (http://www.yeastgenome.org/download-data/) using the SEQUEST search algorithm. Search results were submitted to PeptideProphet [145] and ProteinProphet [146] using TPP and filtered to 1% false positive rate.

Spectra from IP samples were searched using the MSBlender search algorithm against the same protein coding data set. MSBlender searches were carried out using TIDE, MS-GFDB, and InsPecT search algorithms and filtered to a 1% false discovery rate[147].

Generation of protein lists

For each biological replicate LC-MS/MS injections were analyzed independently. Data from multiple injections per biological replicate were combined by summing the spectral counts across injections. All data sets within an experiment were aligned, and proteins observed in only one biological replicate injection were removed. For whole cell lysate samples, the data sets were curated to assign all peptides to a single unique protein or protein group to account for the occurrence of degenerate peptides assigned to multiple proteins (e.g. paralogs). For IP samples, peptide counts were divided evenly among all protein group members sharing that common peptide.

Protein quantification

Proteins were quantified by comparison of spectral counts, as described in Lu *et al.* 2007[144]. Briefly, observed frequencies for each protein were calculated as the of sum spectral counts across biological replicates. Then each protein was given an additional pseudo-count of 1 spectral count to adjust for proteins with a zero-count. Z-scores were calculated for each protein by comparing its relative frequency in the sample to a matched control (e.g. GFP strain vs. untagged control or soluble fraction vs. insoluble fraction). A z-score of $\geq \pm 1.96$ was set as the threshold for significance. Composite *Z*-

scores for fractions were then calculated as the sum individual *Z*-scores per for each biological replicate divided by the square root of the number of Z-scores combined. For the heatshock experiments the differential Z-scores between the 30C and 42C samples were then calculated as the difference between composite Z-scores, again normalized by the number of Z-scores combined (i.e. 2).

Ontology annotation and enrichment calculation

GO term enrichment for among foci forming proteins was calculated with 811 cytoplasmic proteins as a background set with the Bonferroni multiple hypothesis test correction using the BinGO plugin in Cytoscape against the Yeast GO slim database.

Fluorescence activated cell sorting and imaging cells

To determine the effects of increasing cell age on the prevalence of possessing Gln1-GFP foci we grew cultures of Gln1-GFP to stationary phase in YPD (5 days) and fixed as described above. Bud scars on cells were fluorescently marked by the addition of calcofluor white (100µl/ml PBS cell suspension) and incubating 20 minutes. Then, by FAC sorting the brightest 1% of calcofluor white fluorescent cells, we collected a population enriched in old cells. Cells were imaged as described above with the addition capture of calcofluor white fluorescence using a standard DAPI filter set to image bud scars. To improve bud scar and foci imaging, 15 µm deep Z-stacks of 3µm thick slices were captured for each imaged field. Maximum intensity projections were calculated for each Z-stack and, together with the raw data, used to measure cell size (as estimated by

cross sectional area at the focal plane), number of bud scars, and Gln1-GFP foci per cell in the populations.

Measuring the effects of Rapamycin on foci formation by imaging flow cytometry

Gln1-GFP cultures were restarted to 0.2 OD/ml in SC in triplicate and grown to approximately 1 OD/ml in SC before addition of rapamycin (Sigma) in DMSO to reach final concentrations of 200, 100, 20, 1, 0nm rapamycin (DMSO only), and no DMSO. Cells were grown an additional 30h until cultures had passed the diauxic shift before fixing as described for the other foci induction experiments. Cells were imaged on an Amnis ImageStream at 60x in brightfield and GFP channels. For all replicates 50,000 cell images were collected per sample in one session. Images were analyzed in Amnis IDEAS 4.0 software using custom templates to filter images for proper focus, viability, to sort populations by foci phenotype, and quantify various properties of the fluorescence signal, such as the mean max pixel intensity. The percent of cells with foci was calculated as the number of cells with greater than 30% of total cellular GFP fluorescence signal in an area less than approximately 1.25 μm^2. To be precise the gate for the foci size scales with integrated fluorescence intensity (F) by the equation:

$$A = \frac{6\times10^{-5}\,\mu m^2}{\text{flourescence units}} \times F + 1.2\mu m^2$$

where (F) is the integrated fluorescence intensity of smallest number of contiguous pixels that account for at least 30% of total cellular fluorescence and (*A*) is the sum of the area of those pixels.

Author's Contributions

The induction test experiment and the colocalization experiment was the author's work. The heat shock mass spectrometry experiment was the work of the author and Daniel Boutz. The heat shock microscopy was the work of the author and Mark Tsechansky. The co-immunoprecipitation experiments were the work of the author, Ariel Royall and Daniel Boutz. The Western blot shown was the work of Ariel Royall. The scoring of foci vs. cell size was the work of Margerita Driga-West. The FACS experiments were the work of the author. The rapamycin experiment was the work of the author and Mark Tsechansky.

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SUPPORTING INFORMATION

GO Term	p-Value	Genes/term
cellular amino acid metabolic process [GO:0006520]	3.00E-15	43
cellular amine metabolic process [GO:0044106]	4.80E-15	44
amine metabolic process [GO:0009308]	1.68E-14	45
carboxylic acid metabolic process [GO:0019752]	2.19E-13	48
oxoacid metabolic process [GO:0043436]	2.19E-13	48
organic acid metabolic process [GO:0006082]	2.43E-13	48
cellular ketone metabolic process [GO:0042180]	7.26E-13	49
small molecule metabolic process [GO:0044281]	2.03E-09	64
cellular nitrogen compound biosynthetic process[GO:0044271]	1.07E-07	35
regulation of translation [GO:0006417]	2.72E-05	18
tRNA aminoacylation for protein translation[GO:0006418]	6.03E-05	11
amine biosynthetic process [GO:0009309]	7.55E-05	21
glutamine metabolic process [GO:0006541]	1.18E-04	9
amino acid activation [GO:0043038]	1.42E-04	11

tRNA aminoacylation [GO:0043039]	1.42E-04	11
cellular amino acid biosynthetic process [GO:0008652]	2.54E-04	20
organic acid biosynthetic process [GO:0016053]	2.66E-04	23
carboxylic acid biosynthetic process [GO:0046394]	2.66E-04	23
posttranscriptional regulation of gene expression[GO:0010608]	3.40E-04	18
small molecule biosynthetic process [GO:0044283]	1.81E-03	26
glutamine family amino acid metabolic process[GO:0009064]	2.14E-03	12
IMP biosynthetic process [GO:0006188]	4.75E-03	6
IMP metabolic process [GO:0046040]	4.75E-03	6
nucleoside monophosphate biosynthetic process[GO:0009124]	6.61E-03	9
regulation of translational initiation [GO:0006446]	8.02E-03	7
purine ribonucleoside monophosphate biosynthetic process [GO:0009168]	8.02E-03	7
purine nucleoside monophosphate biosynthetic process [GO:0009127]	1.14E-02	7
purine ribonucleoside monophosphate metabolic process [GO:0009167]	1.14E-02	7
biosynthetic process [GO:0009058]	1.17E-02	100
nucleobase-containing compound biosynthetic process [GO:0034654]	1.18E-02	15
ribonucleoside monophosphate biosynthetic process[GO:0009156]	1.27E-02	8
regulation of cellular protein metabolic process[GO:0032268]	1.29E-02	20
nucleoside monophosphate metabolic process[GO:0009123]	1.31E-02	9
nucleotide biosynthetic process [GO:0009165]	1.44E-02	14
purine nucleoside monophosphate metabolic process[GO:0009126]	1.60E-02	7
ribonucleoside monophosphate metabolic process[GO:0009161]	1.65E-02	8
translational initiation [GO:0006413]	2.50E-02	11
regulation of protein metabolic process [GO:0051246]	2.72E-02	20
protein refolding [GO:0042026]	3.08E-02	6

Table S2.1. GO enrichment for foci forming proteins

Systematic name	Gene Name	Systematic name	Gene Name	Systematic name	Gene Name	Systematic name	Gene Name
YMR120C	ADE17	YER023W	PRO3	YIL052C	RPL34B	YIL069C	RPS24B
YMR300C	ADE4	YGL062W	PYC1	YDL191W	RPL35A	YGR027C	RPS25A
YGR061C	ADE6	YBR218C	PYC2	YDL136W	RPL35B	YLR333C	RPS25B
YMR169C	ALD3	YLR075W	RPL10	YLR325C	RPL38	YGL189C	RPS26A
YPR145W	ASN1	YPR102C	RPL11A	YPR043W	RPL43A	YER131W	RPS26B
YGR124W	ASN2	YGR085C	RPL11B	YBR031W	RPL4A	YOR167C	RPS28A
YDR168W	CDC37	YDL082W	RPL13A	YDR012W	RPL4B	YLR264W	RPS28B
YCR005C	CIT2	YMR142C	RPL13B	YML073C	RPL6A	YJR145C	RPS4A
YLR216C	CPR6	YKL006W	RPL14A	YLR448W	RPL6B	YHR203C	RPS4B
YML070W	DAK1	YHL001W	RPL14B	YGL076C	RPL7A	YPL090C	RPS6A
YHR068W	DYS1	YIL133C	RPL16A	YHL033C	RPL8A	YBR181C	RPS6B

YPL037C	EGD1	YKL180W	RPL17A	YLL045C	RPL8B	YNL096C	RPS7B
YMR250W	GAD1	YJL177W	RPL17B	YGL147C	RPL9A	YBL072C	RPS8A
YDL215C	GDH2	YNL301C	RPL18B	YDL130W	RPP1B	YER102W	RPS8B
YPR160W	GPH1	YBL027W	RPL19B	YOL039W	RPP2A	YPL081W	RPS9A
YKL152C	GPM1	YPL220W	RPL1A	YMR230W	RPS10B	YBR189W	RPS9B
YLR192C	HCR1	YGL135W	RPL1B	YDR025W	RPS11A	YOR007C	SGT2
YCL030C	HIS4	YMR242C	RPL20A	YBR048W	RPS11B	YAL005C	SSA1
YLL026W	HSP104	YOR312C	RPL20B	YMR143W	RPS16A	YNL209W	SSB2
YPL240C	HSP82	YBR191W	RPL21A	YDL083C	RPS16B	YLR150W	STM1
YFR053C	HXK1	YPL079W	RPL21B	YML024W	RPS17A	YBR126C	TPS1
YBL076C	ILS1	YLR061W	RPL22A	YDR447C	RPS17B	YML100W	TSL1
YLR432W	IMD3	YGL031C	RPL24A	YDR450W	RPS18A	YJR103W	URA8
YML056C	IMD4	YGR148C	RPL24B	YML026C	RPS18B	YJR014W	YJR014W
YOR232W	MGE1	YLR344W	RPL26A	YML063W	RPS1B	YOR251C	YOR251C
YJL042W	MHP1	YGR034W	RPL26B	YKR057W	RPS21A	YPL009C	YPL009C
YER165W	PAB1	YDR471W	RPL27B	YJL136C	RPS21B	YNL241C	ZWF1
YGR087C	PDC6	YOR063W	RPL3	YGR118W	RPS23A		
YGR240C	PFK1	YLR406C	RPL31B	YPR132W	RPS23B		
YMR205C	PFK2	YOR234C	RPL33B	YER074W	RPS24A		

Table S2.2. Roughly a third of detectable cytoplasmic proteins (117/395) became more insoluble following a 2hr heatshock.

(95% confidence level, Z-score $\geq$1.96)

Systematic Name	Gene Name
YGL234W	ADE5,7
YOR335C	ALA1
YGL105W	ARC1
YDR127W	ARO1
YGR155W	CYS4
YPR035W	GLN1
YCL030C	HIS4
YMR186W	HSC82
YDR171W	HSP42
YPL240C	HSP82
YER165W	PAB1
YDR450W	RPS18A
YHL034C	SBP1

Systematic Name	Gene Name
YAL005C	SSA1
YDL229W	SSB1
YNL209W	SSB2
YKL081W	TEF4
YIL078W	THS1
YJL130C	URA2
YGR094W	VAS1
YGR234W	YHB1

Table S2.3. Among the 50 cytoplasmic proteins that co-immunoprecipitate with Hsp82-GFP, a significant number, 21, also form foci.

($p<4.0E-3$; 97.7% confidence level, Z-score ≥ 2.0)

Systematic Name	Gene Name	Systematic Name	Gene Name
YOR335C	ALA1	YMR309C	NIP1
YOL058W	ARG1	YER165W	PAB1
YDR127W	ARO1	YGR240C	PFK1
YDR380W	ARO10	YMR205C	PFK2
YGL202W	ARO8	YDL055C	PSA1
YPR145W	ASN1	YGL062W	PYC1
YGR124W	ASN2	YBR079C	RPG1
YGR142W	BTN2	YHL033C	RPL8A
YOL139C	CDC33	YOL121C	RPS19A
YAL012W	CYS3	YHR021C	RPS27B
YDL160C	DHH1	YOR167C	RPS28A
YJR137C	ECM17	YLR388W	RPS29A
YPL037C	EGD1	YBL072C	RPS8A
YHR193C	EGD2	YER043C	SAH1
YKL182W	FAS1	YLR180W	SAM1
YAL035W	FUN12	YOR184W	SER1
YER025W	GCD11	YIL074C	SER33
YGL195W	GCN1	YAL005C	SSA1
YFR009W	GCN20	YNL209W	SSB2
YDL171C	GLT1	YPL106C	SSE1

YBR121C	GRS1	YHR064C	SSZ1
YCL030C	HIS4	YLR150W	STM1
YIL116W	HIS5	YBR143C	SUP45
YER052C	HOM3	YKL081W	TEF4
YLL026W	HSP104	YGR162W	TIF4631
YLR432W	IMD3	YBL039C	URA7
YPL145C	KES1	YBR025C	YBR025C
YER036C	KRE30	YDR071C	YDR071C
YGR264C	MES1	YGR285C	ZUO1
YJR010W	MET3		

Table S2.4. Arsenic stress induces aggregates among 59 cytoplasmic proteins

Aggregation of individual proteins was measured by an insoluble shift in fractionated whole cell lysate relative to unstressed control in shotgun mass spectrometry by Jacobson *et al.* 2012.

Chapter 3: Protein aggregation as a widespread and conserved part of aging

INTRODUCTION

Age dependent neurodegeneration is often cause by the buildup of aggregated proteins. In these diseases aggregated proteins, such as polyglutamine in Huntington's or A-beta in Alzheimer's, accumulate within nerve cells in the brain leading to nerve cell death [148],[149]. Additionally, non-disease associated proteins have been detected co-aggregating with disease causing aggregates [150], [151], suggesting an interplay between general protein aggregation and disease specific protein aggregation. While specific disease associate protein aggregates have been shown to cause cell death in across species [125], [148], [152], general protein aggregation during normal remains largely unstudied [153].

As organisms age the processes controlling proteostasis deteriorate. Chaperone expression often decreases with age [121]. Autophagy and proteasome activity decline as well, reducing protein degradation rates in older cells [123]. Older animals also accumulate oxidatively damaged proteins [154] which resist degradation and inhibit proteasomes [94], [155]. Finally, there is evidence from a study of bacterial inclusion bodies that protein aggregation precedes oxidation [156]. Together these findings suggest proteins in general may be more likely to aggregate with age.

A recent study in *Caenorhabditis elegans* measured age dependent increases in protein aggregation [125]. The amount of several hundred insoluble proteins increased in adult worms as they aged. The authors confirmed the buildup of insoluble proteins was due to protein aggregation and furthermore, demonstrated an increase in aggregation of non-disease associated proteins enhanced the toxicity of a disease associated protein.

Widespread protein insolubility and aggregation increases in normally aging worms, raising the possibility that general protein aggregation is an inherent part of aging which may contribute to the onset of neurodegenerative diseases.

We hypothesized that if protein aggregation is indeed a general mechanism of cellular aging, we should observe an increase in protein aggregation in other organisms, such as aging yeast cells. To test this hypothesis we measured the solubility of proteins in yeast growing in liquid culture over the course of 60 hours by shotgun gun mass spectrometry. Protein insolubility increasing with age can be used to profile age dependent aggregation [25]. In accordance with our hypothesis, we observed widespread protein insolubility in the aging yeast; over 15 percent of cytoplasmic proteins were insoluble at any point from late log phase into stationary phase. Interestingly, the particular proteins in the insoluble fraction generally changed with time.

We identified 90 cytoplasmic proteins that become progressively more insoluble with age in yeast. We then compared this set of proteins with a similar set of age dependent protein aggregates discovered in *C. elegans* discovered by David et al. [125] to look for conserved protein aggregation propensities. Indeed, a significant number of orthologous proteins were shared between both sets. Our data provides the first known evidence of conservation of age dependent protein insolubility in normal cellular aging.

Finally, we looked for correlations between protein aggregation and longevity by bioinformatic analysis of co-occurring phenotypes in genetic screens in yeast. I.e., can a knock-down phenotype associated with a particular set of genes predict the genes associated with longevity? In our most significant result, we discovered that genetic manipulations increasing thermotolerance in yeast are likely to also increase lifespan. Our results suggest that cellular mechanisms that confer protein stability have potential to increase longevity.

RESULTS AND DISCUSSION

Measuring protein insolubility in yeast during aging.

Yeast cultures grow logarithmically as long as glucose is available to fuel rapid energy production, but as the glucose runs out cells are forced to transition to a reparative oxidation of ethanol. This change in metabolism, called a diauxic shift, can be observed as an inflection in the growth rate followed by reduced growth rate. Following the diauxic shift, cells that have budded once or less will typically transition to a quiescent state, which should be roughly 50-75% of cells in the culture, hence the reduced growth rate. Cells that do not transition to quiescence continue to bud until they die, but their subsequent daughter cells are born quiescent, further diluting the replicatively aged cells within the population [82]. Thus the onset of chronological aging in yeast begins following the diauxic shift.

To measure changes in protein solubility with aging, we measured the relative solubility of proteins in yeast at late log phase (4h), past the diauxic shift at the start of chronological aging (24h), after 5 days of culturing (60h), and following 2 hours in fresh media (re-fed, 62h) (Figure 1A). Systematic protein solubility profiling was measured by the relative partitioning of each protein between a soluble and insoluble phase at each time point (Figure 1A). We showed previously that insoluble shifts detected by this method correlate well with protein assembly formation of cytoplasmic proteins [1], many of which are aggregates (Chapter 2).

Mass spectrometry profiling of soluble and insoluble fractions produced a comprehensive survey of yeast proteins. Over 4000 proteins were detected throughout the time course, representing greater than two thirds coverage of all open reading frames in *S.*

cerevisiae, with over 1000 proteins seen at all 4 time points (Figure 1B). To calculate the bias of each protein toward the soluble or insoluble fraction, we employed the APEX method [157]. In brief, the difference between the normalized frequency of a protein's spectra observed in the soluble fraction and the normalized frequency of the same protein's spectra observed in the insoluble fraction produced a Z-score defining the confidence of differential partitioning.

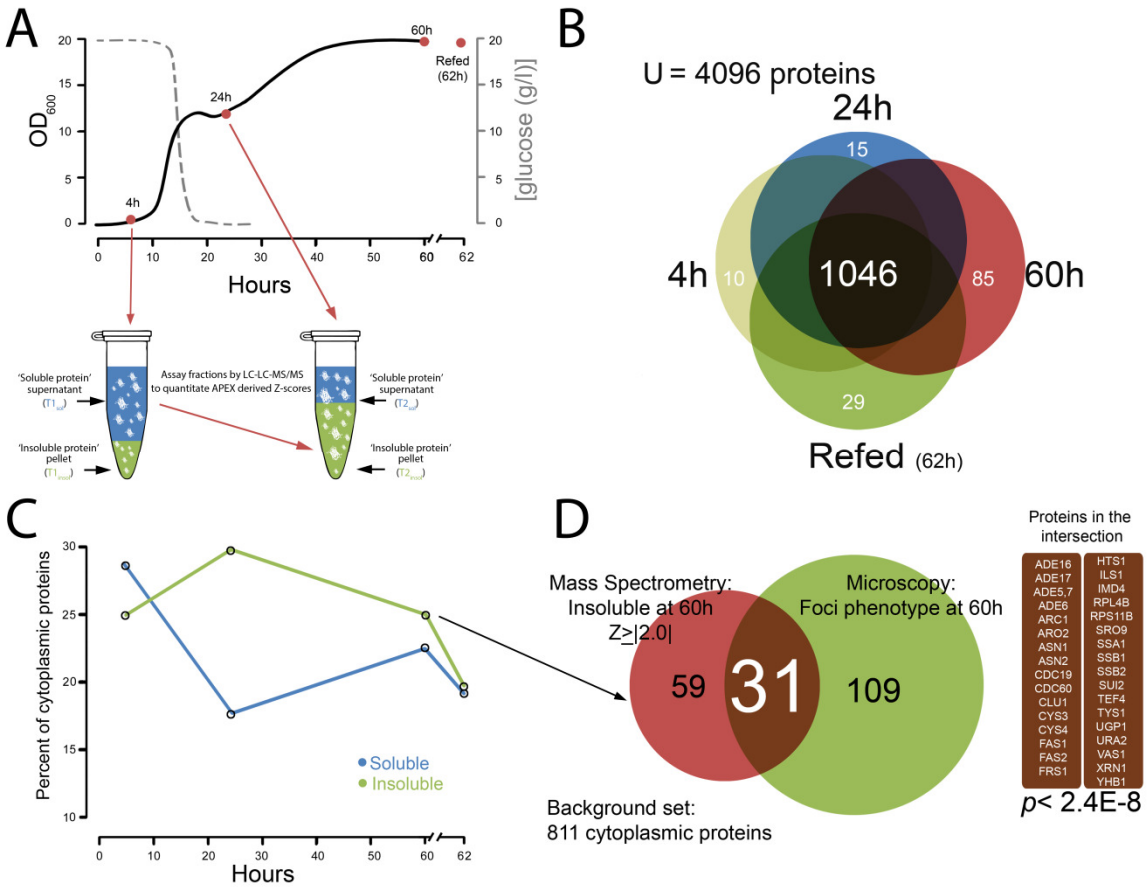

Figure 3-1. Extensive protein insolubility occurs in yeast even under normal growth conditions.

(A) An illustration of the experimental setup for measuring protein solubility over time. Cultures of BY4741 growing in YPD were sampled in late log phase, past the diauxic shift at the entrance to stationary phase, after 5 days of growth, and following 2 hours in fresh media. Cell lysate from each time point was separated into soluble and insoluble fractions and the proteins in each fraction were systematically analyzed by shotgun mass spectrometry to measure the relative solubility of proteins at each time point. (B) Mass spectrometry profiling achieved relatively comprehensive coverage of the yeast proteome, with 4096 out of ~6000 total possible yeast proteins observed in one or more time points and over 1000 proteins observed across all 4 time points. (C) A plot of the percent of cytoplasmic proteins significantly enriched in either the soluble or insoluble at each time point shows a widespread shift of proteins toward the insoluble fraction at the 24h time point. Surprisingly, the solubility of cytoplasmic proteins in general increased into stationary phase (60h) and the percent of both soluble and insoluble proteins dropped following re-feeding cells with fresh media for 2h. (D) A significant overlap (Cumulative

hypergeometric $p < 2.4E-8$) exists between the set of cytoplasmic proteins that are insoluble at 60h with those that formed foci when GFP-tagged at 60h.

Plotting the global trends in cytoplasmic protein solubility shows the largest percentage of proteins insoluble at 24h, with ~30% of cytoplasmic proteins significantly insoluble (Figure 1C). This time point occurs just after the diauxic shift, when the rapid energy production of glycolysis ceases and cells transition to oxidative respiration. Thus the observed spike in protein insolubility could be attributed to the stress of energy depletion, a topic discussed in the next chapter. In support of this idea, we see significant enrichment for protein re-folding machinery in the insoluble phase at 24h (Figure 2A, C).

The global trend between 24h and 60h was an increase in the solubility of cytoplasmic proteins; the percent of soluble cytoplasmic proteins increased while the percent of insoluble cytoplasmic proteins decreased (Figure 1C). This is somewhat surprising as we expected proteins to generally become more insoluble with age. However, there were a set of proteins that, in contrast to the general trend, became increasingly insoluble with age. Of these, a set of 61 proteins became increasingly insoluble from late log phase to 60h but increased in solubility with the addition of nutrients (Figure 2B). This set was enriched for proteins involved in amino acid metabolism and translation (Figure 2D). Looking specifically at the proteins insoluble at 60h, we see a significant enrichment for proteins that formed foci in our previous screen of the GFP collection under the same conditions (Figure 1D) [1].

To assay which proteins where irreversibly insoluble, we re-fed the 60h cultures of cells with fresh media. The addition of fresh media, and particularly additional glucose, causes the dissipation of stationary phase bodies, and return to solubility for many proteins [1], [158], [159]. While most cytoplasmic proteins that became insoluble during aging showed an increase in relative solubility following the transfer to fresh

media, 20 proteins did not. Most of these (14/20) were translation associated proteins by Gene Ontology (GO) term annotation for biological process. In fact, translation associated proteins were the only class of proteins to be progressively more insoluble throughout the experiment, suggesting they are particularly prone to insolubility.

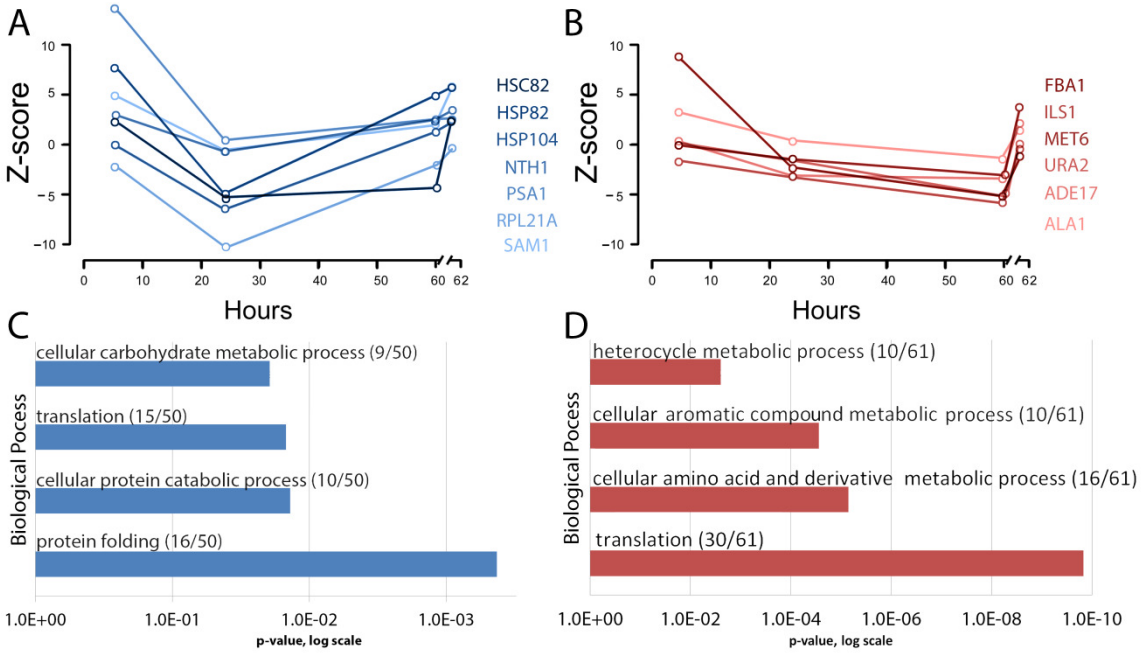

Figure 3-2. The set of insoluble cytoplasmic proteins in yeast cells changes over time. (A) Comparing each protein's Z-score across time points revealed some cytoplasmic proteins' nadir of solubility occurred at 24h followed by a progressive increase in solubility. (B) Another set of cytoplasmic proteins became increasingly insoluble up to 60h as measured by decreasing Z-scores over time. (C) Functional enrichment tests of these 50 proteins for Gene Ontology (GO) annotations were carried out with the hypergeometric test against the background set of 4096 proteins detected in this experiment. A total of 33 out of 50 proteins fell into one or more significantly enriched biological process categories, most notably "protein folding". (D) A total of 43 out of 61 proteins most insoluble at 60h fell into one or more significantly enriched biological process categories using the method detailed in (C), most notably "translation".

One caveat of the method is that mass spectrometry sampling is inherently biased toward observing proteins with higher concentrations. This bias further increases when

restricting analysis to proteins seen in multiple time points for comparing solubility over time. This method may especially underestimate the amount of aggregation in cells, as the most aggregation prone proteins are predicted to have the lowest expression levels [106][160].

Conservation of age dependent protein insolubility between yeast and worm

Having found a widespread increase in protein insolubility in yeast, we then tested for conservation of the phenomenon. The first and so far only other published experiment measuring general protein aggregation with age was done in *C. elegans* by David et al. [125]. David and colleagues found that the amount of insoluble proteins increased in adult worms as they aged from 3 days to 10 days old. They used a genetic trick to produce worms lacking active germline cells, so all cells in the worms were post mitotic and aging only chronologically. They lysed the worms at the 3 and 10 days and extracted the detergent insoluble proteins for comparison by mass spectrometry. They found several hundred proteins from many cellular compartments and tissue types increased in the insoluble fraction between the two time points. As further validation that the accumulation of insoluble proteins represents aggregation, they demonstrated several fluorescent-tagged proteins transitioned from diffuse to foci localization in older animals.

To compare the set of insoluble proteins found in worms to the set we found in yeast, we restricted both sets to orthogroups common between the two species using orthogroup mappings from www.phenologs.org [161]. We were left with 226 worm proteins with orthologs in yeast and 90 cytoplasmic yeast proteins with orthologs in worm, with 35 orthologs shared by both sets (Table S3-2). A hypergeometric test shows this overlap is highly unlikely to occur by random chance ($p < 2.4E-11$), indicating

conservation of age dependent aggregation between the two species. We speculate that age dependent aggregation may in fact be a general property of aging, conserved across all species that exhibit senescence. A total of 27 out of the 35 proteins were annotated with "translational" GO biological processes, a significant enrichment among the set of proteins in the overlap (hypergeometric p < 2.6E-22). We rationalize that translation associated proteins might be evolutionarily trapped in sequence space at a high aggregation propensity in order to preserve their very necessary cellular function. Alternatively, increased exposure to unfolded nascent chains is an inherent occupational hazard of ribosomal proteins, which may increase the rate they are nonspecifically incorporated into protein aggregates.

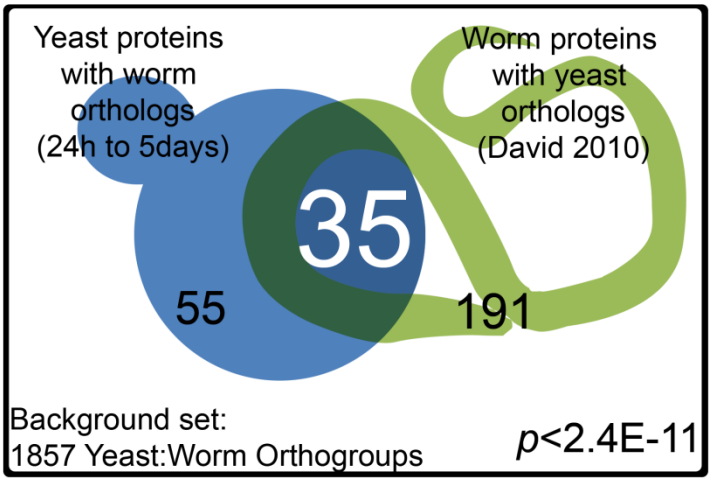

Figure 3-3. Increasing aggregation of particular proteins with age is conserved between *S. cerevisiae* and *C. elegans*.

To compare between organisms we restricted the sets of insoluble proteins to only those with orthogroup mappings in both yeast and worm [161]. In yeast the mapping defined a subset of 90 proteins that became more insoluble between 24h and 60h with an ortholog in worms. From the worm data of David et al. [125], the mapping produced a set of 226 worm proteins (with a yeast ortholog) that became more insoluble between day 3 and day

10. A significant overlap exists between the two sets, as evidenced by a cumulative hypergeometric $p< 2.4E-11$.

A genetic link between thermotolerance and aging supports general aggregation as a common mechanism of senescence

Data from deletion and loss of function screens posted to the Saccharomyces Genome Database was curated to pool various measures of thermotolerance and lifespan across experiments. A significant overlap was found between thermotolerance and increased lifespan (hypergeometric $p<7.3E-3$), and an even more significant overlap between thermotolerance and increased chronological lifespan in particular (hypergeometric $p<5.3E-5$) (Figure 3-4). In contrast, none of the genetic perturbations that increased thermotolerance are known to reduce lifespan. Association rule mining extracted directed relationships between these pooled sets and found that increased thermotolerance is predictive of an increase in lifespan and chronological lifespan, as indicated by the confidence scores.

Our results suggest that cellular mechanisms that confer protein stability have potential to increase longevity. In fact, many of the mechanisms that increase thermotolerance either prevent protein aggregation, such as increased chaperone expression [162] and trehalose production [115], or degrade protein aggregates, such as autophagy [163][164]. Thus mechanisms that reduce general protein aggregation may have the potential to influence lifespan.

Interestingly, the complete lack of overlap between genetic perturbations that increased thermotolerance and any measure of reduced lifespan suggests attempting to

increase lifespan through improved thermotolerance may be a safer route to longer life than current pharmaceutical interventions, such as rapamycin, which suppresses immune function.

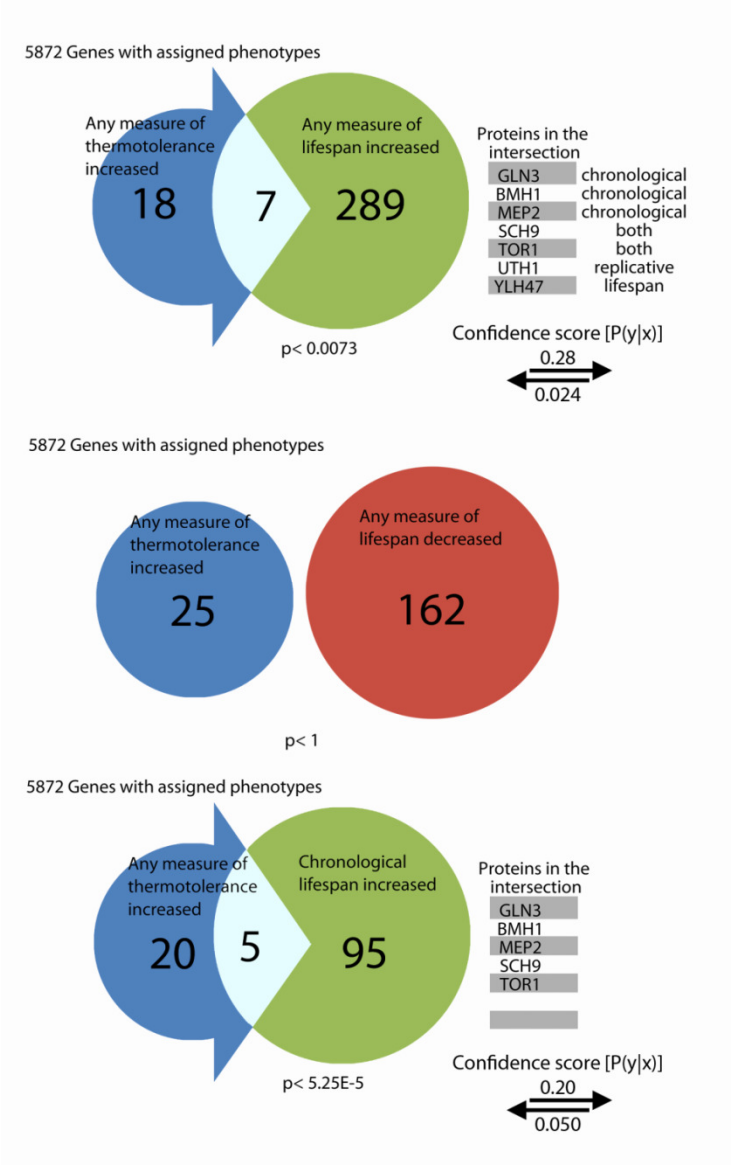

Figure 3-5. Thermotolerance is predictive of lifespan increase, particularly chronological lifespan.

CONCLUSION

The previous chapter argued that many foci are protein aggregates and, based on Gln1-GFP as a case study, their occurrence increases with both chronological and replicative age. This chapter builds on that case to argue that age dependent protein aggregation is a general property of many, if not all, cytoplasmic proteins and that this property is conserved across different species. We showed that protein insolubility is a common aspect of normal yeast growth, but the particular proteins that are insoluble generally changes with time. Within these data, we identified 90 cytoplasmic proteins that become more insoluble with age. These yeast proteins are orthologs of a similar set of proteins in *C. elegans* that also become increasingly insoluble with age, providing the first evidence that age dependent insolubility propensity is conserved. We conclude that widespread protein insolubility occurs even in normal conditions in aging yeast, and that this insoluble propensity with age is a conserved property of these proteins.

Future work analyzing the set of evolutionarily conserved age insoluble proteins for structural motifs or aggregation signatures may be able to find the physiochemical properties responsible for aggregation of this set of proteins. Looking for commonalities between the conserved set found here and proteins that influence or co-aggregate with human diseases aggregates as evidence for an evolutionarily ancient protein aggregation propensity influencing human cellular senescence.

METHODS

Media and yeast strains

Yeast strains had a genetic background of BY4741 [genotype, MATa *his3_1 leu2_0 met15_0 ura3_0*]. Rich (YPD) medium containing Yeast Extract (1%), Peptone (2%) and glucose (2%) was purchased from Sunrise Sciences. Cultures were started from by picking from freezer stocks into YPD and growing overnight before transferring to new media for regrowth. All cultures were maintained shaking at 30°C unless otherwise specified.

Liquid Culture Time Course

Yeast cultures were for the time course were started as described above to an initial optical density (O.D.) of 0.2 per ml in 500ml YPD as a pair of biological replicates. Sampling of cultures took place ~ 4h after starting when cells reached late log phase (1 O.D./ml), at 24h once cells had passed the diauxic shift, at 60h, and again after resuspending cells in fresh media for 2h. At each time, we measured the O.D. and withdrew culture media containing 100 O.D. worth of cells (e.g 100ml at the first time point). Centrifuging cells for 5 minutes at 1000x g produced a loose cell pellet. The pellet was washed in lysis buffer, lysed by bead beating, and fractioned as described in for the heat shock experiment in the preceding chapter.

Mass spectrometry

Preparing proteins for mass spectrometry analysis proceeded precisely as described in the preceding chapter for the LTQ-Orbitrap XL (Thermo) .

Bioinformatic analysis of peptides

Spectra from the whole cell lysate were searched against the non-redundant yeast protein-coding data set downloaded from SGD (http://www.yeastgenome.org/download-data/) using the SEQUEST search algorithm. Search results were submitted to PeptideProphet [145] and ProteinProphet [146] using TPP and filtered to 1% false positive rate. Spectra were searched using the MSBlender search algorithm against the same protein coding data set. MSBlender searches were carried out using TIDE, MS-GFDB, and InsPecT search algorithms and filtered to a 1% false discovery rate[147].

Generation of protein lists

For each biological replicate LC-MS/MS injections were analyzed independently. Data from multiple injections per biological replicate were combined by summing the spectral counts across injections. All data sets within an experiment were aligned, and proteins observed in only one biological replicate injection were removed. For peptide counts matching to paralogs or protein groups sharing tryptic sequences, peptide counts were divided evenly among all protein group members sharing the common peptide.

Protein quantification

Proteins were quantified by comparison of spectral counts, as described in Lu et al. 2007[144]. Briefly, observed frequencies for each protein were calculated as the of sum spectral counts across biological replicates. Then each protein was given an additional pseudo-count of 1 spectral count to adjust for proteins with a zero-count. Z-scores were

calculated for each protein by comparing its relative frequency in the sample to a matched control (e.g. GFP strain vs. untagged control or soluble fraction vs. insoluble fraction). A *z*-score of $\geq \pm 1.64$ (90% confidence interval) was set as the threshold for significance. Composite *Z*-scores for fractions were then calculated as the sum individual *Z*-scores per for each biological replicate divided by the square root of the number of Z-scores combined. For the comparing between time points experiments, differential Z-scores were then calculated as the difference between composite Z-scores, again normalized by the number of Z-scores combined (e.g. proteins more insoluble over between 24h and 60h would be calculated as $\Delta Z_{60h\text{-}24h} = Z_{60h} - Z_{24h}$).

Ontology annotation and enrichment calculation

GO term enrichment for among foci forming proteins was calculated with the 4096 proteins detected in this set of experiments as a background set with the Bonferroni multiple hypothesis test correction using the BinGO plugin in Cytoscape against the Yeast GO slim database. GO term enrichment was calculated similarly for conserved aggregating proteins but using the 1857 *C. elegans* orthogroup mappings to *S. cerevisiae* as the background set.

Ortho-group mapping of aggregating proteins between *S. cerevisiae* and *C. elegans*

Ortholog matching between yeast and worm proteins used the orthogroup mapping developed by McGary et al. 2010. From our yeast data we selected the set of cytoplasmic

proteins that became relatively more insoluble between 24h to 60h to compare with the set of proteins David et al. 2010 observed an increase in counts in the insoluble fraction between young and old worms. Protein sets were mapped to the orthologs common between yeast and worm using http://www.phenologs.org/ [161]. This produced subsets of the insoluble proteins from each experiment that have orthogroup mappings between worm and yeast. To compare the significance of the similarity between the sets we calculated the hypergeometric probability of the overlap using the 1857 Ce-Sc.Sc orthogroups from http://www.phenologs.org/as the background.

Association rule mining of genetics screens for relationships between thermotolerance and lifespan in yeast

Original SGD phenotypes were downloaded as phenotype_data.tab from http://www.yeastgenome.org/download-data/curation in June 2012. Phenotypes were processed to include only those resulting from "null" or "reduction of function" mutations, and to include only ORFs. Additional processing was performed to consolidate all related 'heat sensitivity' and 'thermolerance' phenotypes. Association rules were mined from these processed phenotypes. To find rules, gene-phenotype associations were formatted to treat each gene as a transaction, and their associated phenotypes as 'purchased' items. Rules associating phenotypes were then obtained using the R package 'arules' with the 'apriori' algorithm. An item set size limit of two phenotypes was used for rule discovery. The confidence score of each rule was used to assess directionality. Confidence scores were calculated as the frequency of proteins in

the intersection divided by the total proteins in the source set. For example, conf(chronological longevity→thermotolerance) = supp(longevityUthermotolerance)/sup(longevity) = 5/100 = 0.05. To measure significance of overlap without regard to directionality, the cumulative hypergeometric probability of the overlap for each rule was computed.

Author's contributions

The association-rule mining experiment was designed by Mark Tsechansky and Jon Laurent and executed by Jon Laurent. All other work was the author's.

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SUPPLEMENTAL INFORMATION

Systematic Name	Gene
YLR388W	RPS29A
YCR077C	PAT1
YGR189C	CRH1
YLR257W	
YER052C	HOM3
YOL121C	RPS19A
YNL302C	RPS19B
YHR021C	RPS27B
YDL082W	RPL13A
YER176W	ECM32
YIL069C	RPS24B
YMR194W	RPL36A
YPL131W	RPL5
YER074W	RPS24A
YGR034W	RPL26B
YOR091W	TMA46
YKL204W	EAP1
YPR163C	TIF3
YOR232W	MGE1
YIL135C	VHS2

Table S2.1. Proteins that become increasingly insoluble in aging yeast and do not remain insoluble after addition of nutrients.

Systematic Name	Gene	Systematic Name	Gene
YJR105W	ADO1	YLR325C	RPL38
YAL003W	EFB1	YPL131W	RPL5
YOR133W	EFT1	YGL076C	RPL7A
YDR385W	EFT2	YPL198W	RPL7B
YOR317W	FAA1	YDR382W	RPP2B
YJR070C	LIA1	YDR064W	RPS13
YOR310C	NOP58	YOL040C	RPS15
YLR029C	RPL15A	YML026C	RPS18B
YMR121C	RPL15B	YOL121C	RPS19A
YNL301C	RPL18B	YNL302C	RPS19B
YBL027W	RPL19B	YER074W	RPS24A
YBL087C	RPL23A	YIL069C	RPS24B
YER117W	RPL23B	YJR123W	RPS5
YBL092W	RPL32	YKL117W	SBA1
YER056C-A	RPL34A	YGR192C	TDH3
YIL052C	RPL34B	YKL056C	TMA19
YDL191W	RPL35A	YOR332W	VMA4
YDL136W	RPL35B		

Table S2.2. Proteins that show a conserved pattern of increasing aggregation with age in both yeast and worm.

Chapter 4: Cellular Energy Buffers Protein Aggregation Propensity: Rappelling over the "edge"

INTRODUCTION

The central importance of amyloidal and prion proteins in neurodegenerative diseases is widely recognized, and the number of diseases linked to protein aggregation is growing continuously [155]. Interestingly, evidence is building that protein aggregation *in vivo* may be far more common than expected, with protein aggregation implicated as the proximal cause of cell death in progeria [165], some types of cardiovascular diseases [166], and stroke [167].

Cells possess many active strategies for controlling protein aggregation, ranging from molecular chaperones to a diverse array of subcellular compartments for storing, degrading, and perhaps recovering aggregated proteins [168][108]. Recent evidence has also begun building for the evolutionary signatures associated with widespread protein aggregation[169][170]. In a particularly seminal paper, Tartaglia *et al.* demonstrated a surprising anti-correlation between the cellular expression levels of a set of eleven human proteins and their aggregation rate *in vitro* [106]. They observed that each protein was naturally expressed *in vivo* at levels strongly correlated to its solubility, defining a solubility boundary which they dubbed the "edge" (**Figure 1**), with highly aggregating proteins maximally expressed at low levels, and less aggregating proteins maximally

expressed at higher levels. This hypothesis suggests that proteins which are likely to form aggregates due to their physiochemical properties, all else being equal, will be constrained to lower abundances than proteins with a lower natural propensity to aggregate. One surprising aspect was that protein expression levels were not simply bounded by the "edge," but rather they were uniformly found to be on the "edge", suggesting a balance of evolutionary selection between protein expression and aggregation propensity.

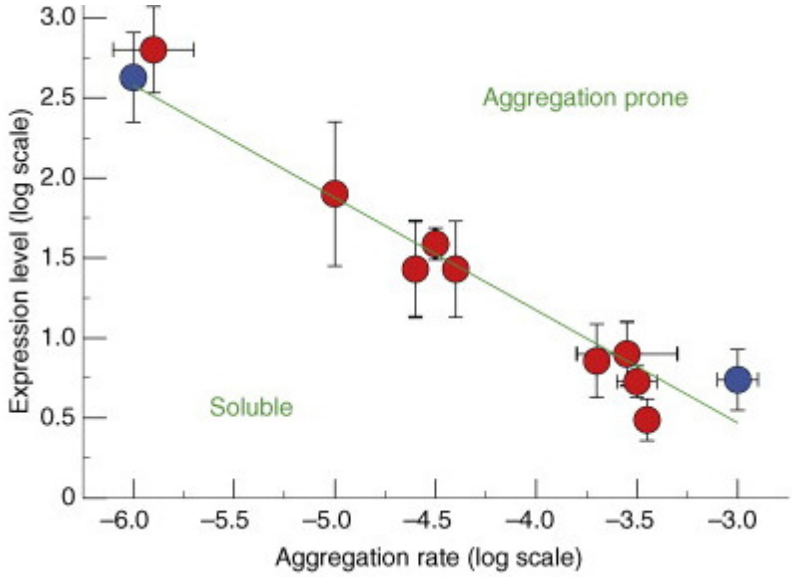

Figure 4-1. "Life on the edge" concept .
Tarteglia et al. demonstrated an inverse relationship between protein concentration, as measured by mRNA expression, and *in vitro* protein aggregation rate. They put forward the idea that cells have evolved to maintain *in vivo* protein aggregation propensity at the limits of solubility or "the Edge" (shown as a green line) (adapted from [106]).

While most (nine) of the proteins initially tested were known to be involved in protein aggregation diseases, more recent evidence suggests that the "edge" hypothesis might apply more broadly than to these largely pathological proteins. Perhaps the strongest support for increased generality of the observation came from a consideration of *E. coli* mRNA abundances, which showed a consistent anti-correlation with aggregation propensity, to the extent that mRNA levels could be in part predicted based upon predicted aggregation propensities [160].

Given each protein's inherent propensity to aggregate, the "edge" (*i.e.*, the abundance level above which proteins will be significantly more likely to aggregate) may not be fixed. Rather, the "edge" may depend upon the state of the cell, specifically its energy state and the availability of agents that are instrumental in dissolving aggregates, such as chaperones, proteasomes, autophagy, and so on. Thus, a simple but seemingly reasonable extension to the "edge" hypothesis would include a consideration of cellular energy state in addition to protein abundance and their intrinsic physicochemical aggregation propensities. For instance, mildly expressing even a non-functional protein past its solubility limit (i.e. YFP with an increased aggregation rate) reduced growth rate by 2.3%, suggesting a fitness cost [143]. We posit this growth rate change results from energy redirected from growth into preventing aggregation and resolubilizing or degrading insoluble proteins.

We wished to test the generality of the "edge" hypothesis and to evaluate whether such factors as physiological and cellular state did in fact play roles in setting the "edge". For these tests, we took advantage of large-scale datasets of protein localization in the budding yeast *S. cerevisiae* and the fission yeast *S. pombe*. Additionally, we used conducted *in vivo* experiments in *S. cerevisiae,* a simple and tractable model of protein aggregation, using microscopy and imaging flow cytometry. In particular, recent systematic microscopy-based screens have been performed under conditions which may to lead to protein aggregation. The first data set looked at ~800 yeast strains from the *S. cerevisiae* GFP-tag collection. These strains, expressed each expressing a GFP-tagged protein from their native locus in the genome, were grown to stationary phase. Normally cytoplasmic proteins are distributed diffusely, but under these conditions 180 proteins involved in intermediary metabolism and stress response formed cytoplasmic foci [1]. The second data set was drawn from a protein localization screen in *S. pombe* of strains expressing inducible YFP-tagged proteins. It too showed a profusion of novel cytoplasmic structures. We analyzed these datasets for evidence supporting the edge hypothesis, then characterized in more detail one particular protein aggregate—the biosynthetic enzyme glutamine synthetase—for the energy dependence of crossing the "edge."

RESULTS AND DISCUSSION

Widespread foci formation in *S. pombe* YFP screen

The cellular distribution of proteins in the fission yeast *S. pombe* has been extensively mapped using libraries of strains expressing a given fluorescently tagged protein [73–76]. While not searching specifically for new structures, such screening efforts have frequently discovered "cytoplasmic dots" in many strains. We argue these structures are widespread aggregation, due in part to the conditions under which localization was measured and the predicted aggregation propensities of foci forming proteins compared to non-foci forming proteins. We focused on the screen by Matsyama et al. 2006 for which the image data, annotations, and expression levels were made publicly available. In this *S. pombe* screen, the strongly inducible nmt1 promoter connected to open reading frames (ORFs) was cloned into the genome at the *leu1* locus with a C-terminal YFP tag to create a library of 4431 clones, each expressing a different YFP labeled protein[73]. We re-analyzed the data for cytoplasmic dots or fibers and found over 500 proteins matching these phenotypes (**Figure 2**). Interestingly, many of these structure-forming proteins are orthologs of structure-forming proteins in *S. cerevisiae* (e.g., CTP synthase and Ade4p [1]).

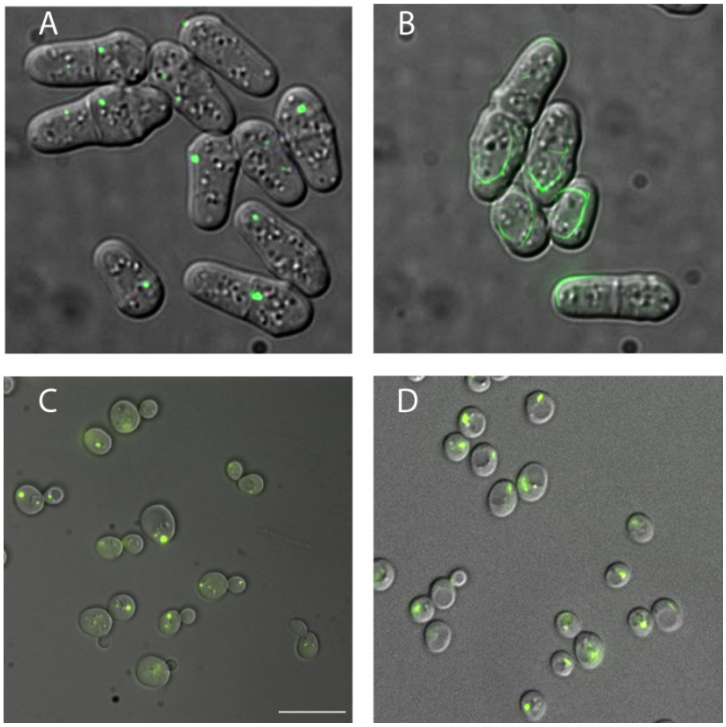

Figure 4-2. Many green or yellow fluorescent protein (YFP or GFP)-tagged proteins formed cytoplasmic structures in *S. pombe* or *S. cerevisiae*, respectively. Fluorescence microscopy images Ade4-YFP foci (A) and Pil1-YFP fibers (B) are representative examples of the phenotypes in *S. pombe*. (adapted from [73]). In *S. cerevisiae* similar phenotypes are seen for Ade4-GFP (C) and Asn2-GFP (D) (adapted from[1], scale bar for C and D is 10μm).

The sets of foci and fiber forming proteins in *S. pombe* and *S. cerevisiae* possess properties of aggregates

Several lines of evidence support the notion that foci from large-scale screens are the result of aggregation we applied the TANGO algorithm [142] to predict the aggregation propensity protein. TANGO employs a statistical mechanics model for predicting aggregation propensities of peptides. This model assumes the possibility of 5

conformations of a given peptide sequence within a small evaluation window and assigns a score to the residue in the center based on the probability of each conformation. The window steps sequentially across the protein sequence to create a profile of aggregation prone regions within a protein. The algorithm was trained on empirical analysis of *in vitro* peptide aggregation and has proved successful in predicting protein aggregation propensity of mutant form of several proteins, and has been widely used for aggregation prediction [171][172]. TANGO scores were elevated for foci-formers in both *S. cerevisiae* and *S. pombe*, suggesting many of the foci represent aggregates (**Figure 3A,** Mann-Whitney U test, $p < 1.0E-4$, and 1.0E-7, respectively).

Consistent with the life-on-the-edge hypothesis, the normal *S. cereviseae* cellular abundances of foci-forming proteins are lower than the non foci-forming proteins (Mann-Whitney U test, $p < 1.0E-8$). Similarly the relative abundances of proteins in the *S. pombe* over-expression screen are lower for foci-forming compared to non foci-forming proteins (Mann-Whitney U test, $p < 1.0E-16$, **Figure 3B**). The trends in both experiments support GFP-protein foci as aggregates as predicted by the "life on the edge" hypothesis of a proteome wide solubility constraint on protein expression.

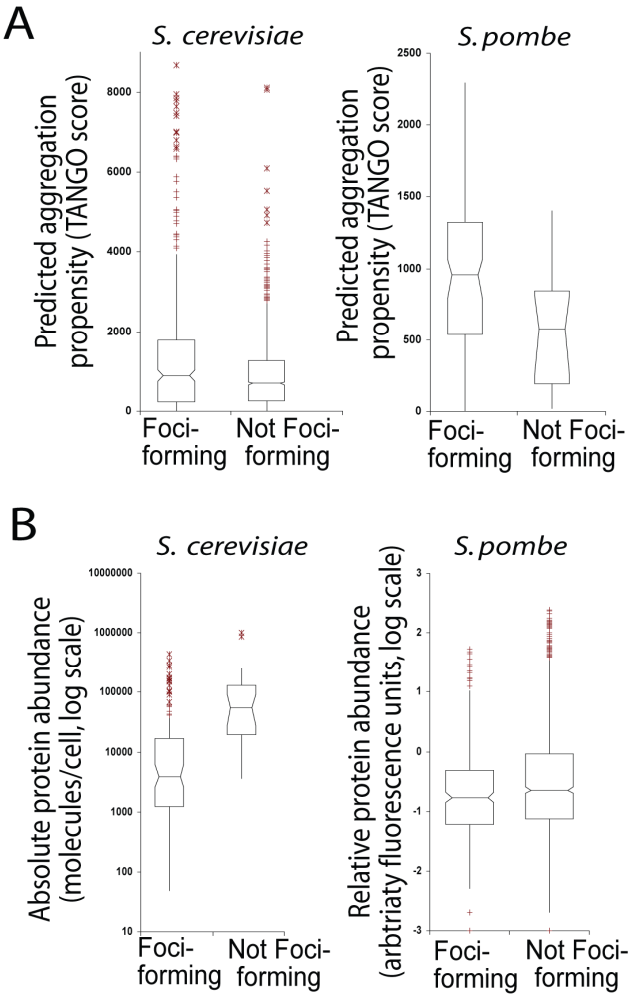

Figure 4-3. Foci forming proteins in *S. cerevisiae* and *S. pombe* show an inverse relationship between abundance and aggregation propensity predicted by "the edge" for general protein aggregation.
(A) Aggregation propensity predicted using the TANGO algorithm for cytoplasmic proteins in *S. cerevisiae* and all proteins in *S. pombe*. The mean TANGO score for foci forming proteins is higher than non-foci forming proteins in both screens which is consistent with the "edge" hypothesis' prediction for aggregate formation. (B) Comparing the mean abundances for foci forming proteins and non-foci forming proteins shows foci forming proteins are expressed at lower concentrations.

The *S. cerevisiae* glutamine synthetase (Gln1-GFP) as a model aggregate

To evaluate the life-on-the-edge phenomenon in more detail, we focused on the yeast glutamine synthetase (Gln1), which synthesizes glutamine from glutamate, ammonia, and ATP. We previously observed Gln1-GFP was diffusely distributed throughout the cytoplasm in log phase yeast cells but robustly formed foci in stationary phase yeast cells [1]. We showed previously (Chapter 3) that Gln1-GFP foci are likely protein aggregates based on their formation following various types of starvation and heat shock and co-immunopurification exclusively with Hsp90 chaperones **(Figure 4).**

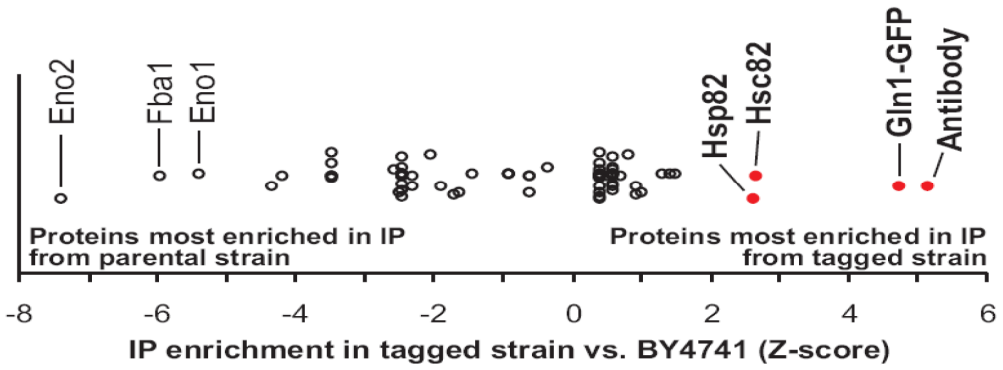

Figure 4-4. Using Gln1-GFP as a model for formation, foci composition was assayed by immunoprecipitation and mass spectrometry analysis.
Z-scores were calculated for each protein based on its relative enrichment in the Gln1-GFP immunoprecipiation samples compared to the untagged controls. Strikingly, the Gln1-GFP pull-down material was dominated almost entirely by glutamine synthetase and one or possibly both of the molecular chaperones Hsp82 and Hsc82. No other proteins were detected a beyond the significance threshold (z-score $\geq$ 2).

Homogeneity is a hallmark of many purified aggregates[173]. Indeed, at high concentrations *in vitro*, *E. coli* glutamine synthetase is known to spontaneously assemble

into filaments [174], suggesting the protein may have an intrinsic propensity to self-organize into macromolecular assemblies, although we see no evidence for filament formation *in vivo* in yeast. While a uniquely identifiable peptide was observed only for Hsc82p, the two HSP90 family chaperones are approximately 97% identical in sequence. Since nearly all observed peptides match to both proteins' sequences, the presence of Hsp82p cannot be ruled out. Members of the HSP90 chaperone family are required for folding difficult-to-fold proteins as well as for refolding denatured proteins after stress [175]. Thus the homogeneity of the Gln1-GFP foci, combined with the strong enrichment for HSP90 chaperones and the observed transition to the insoluble phase, strongly suggests the foci represent mis-folded or misassembled protein aggregates rather than functional assemblies.

Formation of Gln1-GFP foci depends strongly upon cellular energy levels

Gln1 is normally expressed in exponentially growing yeast cells in rich media at very high levels (approx. 350 000 molecules / cell [176]). The life-on-the-edge theory would suggest the protein sits close to the "aggregation edge". Given this high starting level of Gln1, we hypothesized that depleting cellular energy levels would lead to less effective protein homeostasis and a concomitant aggregation of Gln1. Indeed, we have previously observed Gln1-GFP to form foci both in stationary phase cells, but also when cells are transferred into glucose dropout medium **(Figure 5A).**

Using imaging flow cytometry, we quantified the degree of Gln1-GFP foci formation as a function of glucose concentration, measuring the penetrance of foci formation across the population of cells as a function of time **(Figure 5B).** We found that decreasing glucose concentrations led to a corresponding decrease in the onset time of Gln1-GFP foci formation **(Figure 4C,D)**, providing evidence for the energy dependence Gln1-GFP foci formation.

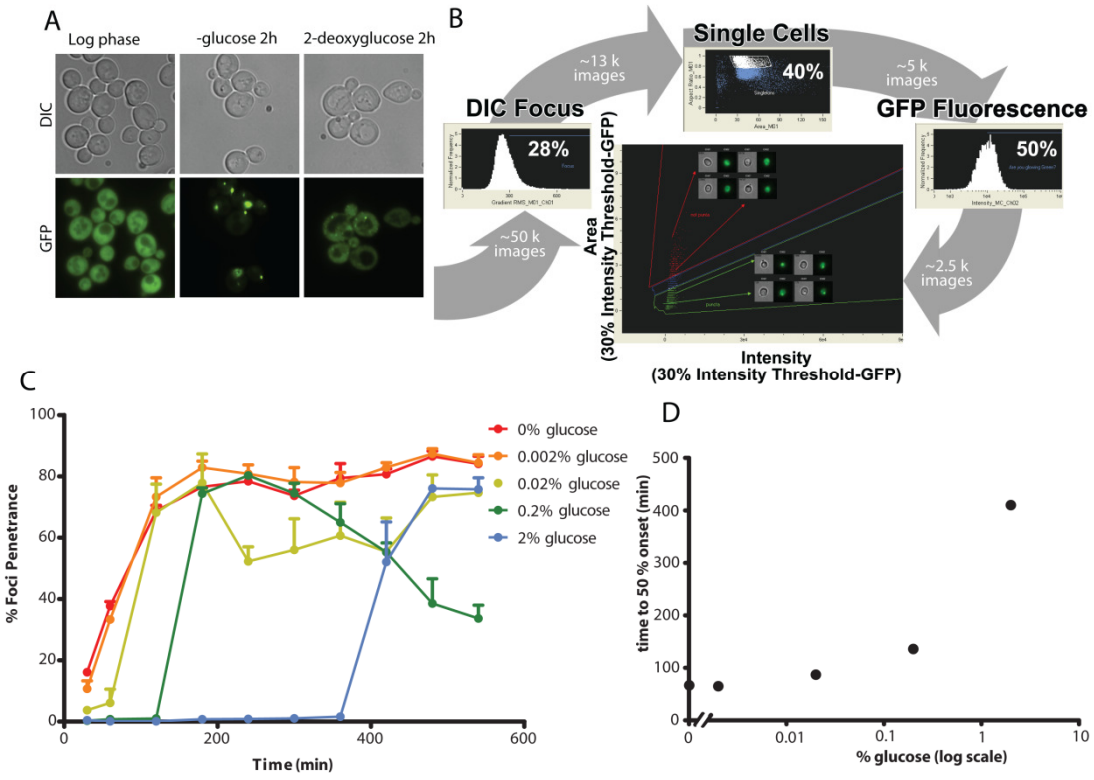

Figure 4.5: Testing the energy dependence of Gln1-GFP aggregate formation. (A) Fluorescence microscopy of Gln1-GFP tagged strains in log phase growth show for when starved for energy either by removing glucose from the growth media or the addition of a non-metabolizable competitive inhibitor, 2-deoxyglucose. (B) Schematic view of foci counting workflow. Approximately 50,000 images are captured per samples on an Amnis ImageStream and processed in an in-house built template to select the most in-focus cells, then individual cells, then cells that show GFP fluorescence. Cells that

pass these filters are graphed based on the intensity and size of the 30% intensity mask which distributes cells into foci and non-foci containing populations. (C) Quantifying foci formation over time across a range of range of glucose concentrations shows a range of kinetics (D) Plotting the concentration of glucose for a culture against the time to 50% foci penetrance reveals a correlation between energy level and the rate of foci formation.

Gln1-GFP aggregates coalescence into single foci in an energy dependent manner

We further tested the energy dependence of protein aggregation by measuring Gln1-GFP foci formation in response to abrupt interruptions of glycolysis, the major source of cellular energy in rapidly growing yeast cultures. Gln1-GFP tagged yeast cells growing exponentially in 2% glucose were transferred to media lacking glucose. In the majority of cells Gln1-GFP formed a few, intense foci as seen in the previous experiment **(Figure 5A)**.

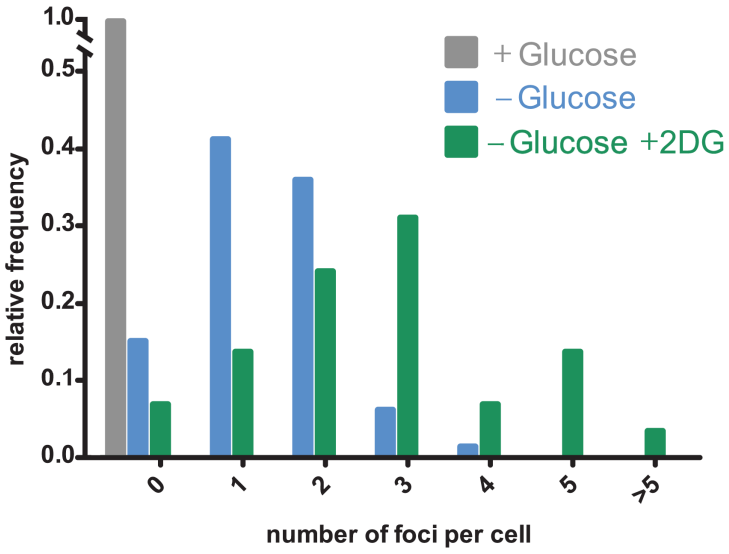

Figure 4-6. Cells contain more Gln1-GFP foci when glycolysis is severely interrupted.
Removing glucose from the media indirectly halts glycolysis, and leads to strong induction of one or two Gln1-GFP foci per cell after 2h. Halting glycolysis by both glucose depletion and the addition of a competitive inhibitor of glucose hexokinase, 2-deoxyglucose (2DG), not only induces foci, but leaves cells peppered with small foci rather than one or two large foci.

Moreover, when Gln1-GFP tagged yeast cells were grown in media lacking glucose and containing 2DG, to mimic a state of extreme starvation, many small foci formed rather than single large foci as commonly seen in glucose depletion alone **(Figure 6).** This suggests that small foci may represent spontaneously forming small oligomers that coalesce into fewer large foci in an energy dependent process. One mechanism could be through active transport, similar to the formation of aggresomes [177],[68]. Another possible mechanism could be through chaperone mediated dynamic partitioning, as seen in bacterial inclusion bodies [178]. In either case the energy dependent assembly of larger structures removes smaller, presumably more toxic aggregates [94] from the

cytoplasm by collecting them into a localized structure to improve cellular fitness. Our observations of Gln1-GFP foci seem to show similar energy dependence for localization, suggesting the cell actively fights the existence for small oligomers.

Extending the life-on-the-edge hypothesis to consider cellular energy state

The "life-on-the-edge" hypothesis insightfully claims that proteins evolved to be at the limit of solubility in their respective cellular environments and that any increase in protein concentration or change in chemistry to increase aggregation rate will lead to insolubility and aggregation. However even in a constant environment cellular energy varies with cell cycle and most environments are far from constant. When cellular energy is high, cells can expend energy through many ATP-dependent protein quality control systems, such as the use of chaperones to force insoluble proteins back across the edge into a soluble state **(Figure 7)**. Aggregates resistant to refolding or targeted degradation can still be removed from the cytosol by actively trafficking them to autophagosomal/lysomal bodies or into a rapidly growing list of aggregate sequestering structures [90][177]. When intracellular energy drops, these mechanisms can no longer functions to buffer proteins that have crossed the edge of solubility, and insoluble proteins will accumulate and begin to oligomerize [179].

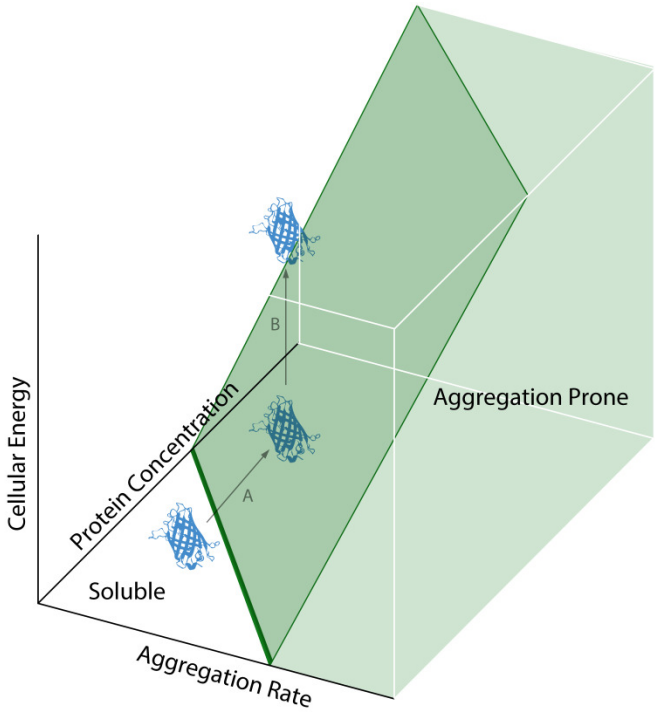

Figure 4-7. **Proposed Model: Extending the "edge" hypothesis**
along the axis of cellular energy creates a new model were proteins' position relative to a surface describes protein solubility/aggregation propensity.

An economic metaphor of deficit spending can be used to understand the effects of cellular energy on protein solubility. Cells in a resource rich environment will spend some of their resources to produce enzymes to acquire more resources from their environments. Expressing enzymes at "the edge" of solubility would be a balanced budget. However cells can acquire more nutrients from the environment faster by expressing enzymes beyond the balance point (**Figure 7**, arrow A) as long they also spend on chaperones to keep them soluble. Proteins beyond the solubility limit create a "solubility debt" whose interest paid is cellular energy expended by chaperones and other proteostasis machinery to maintain solubility despite expression beyond the balance point

(**Figure 7**, arrow B). Growing cells essentially bet that the environment will continue to be good long enough to import and metabolize sufficient nutrients to either pay down the debt or subsidize the interest payments.

The level of "solubility debt" a cell takes on should be proportional to perceived concentration of available nutrients. So there are naturally diminishing returns for expression of any given protein for a finite quantity of metabolite. Also, interfering with cells' ability to sense nutrients should have a similar effect as starvation (e.g. rapamycin treatment, which mimics starvation [180] or caloric restriction [181] depending on the concentration and duration of treatment). Further, the more drastic the change in nutrients (e.g. 2% glucose to 0%), the more unexpected "solubility debt" cells will, on average, be stuck with, leading to faster aggregate build up (which we see for Gln1-GFP, **Figure 5C**) and more cells exiting the cell cycle, which requires efficient proteasome activity to proceed [182]. Indeed, rapid glucose depletion will arrest cells at any point in the cell cycle into G_0 [158]. The model further predicts that the greater the change in nutrients, the more protein should aggregate proportionally extending recovery times to cell cycle re-entry or reducing lower probability of exiting a G_0 arrest. Furthermore, if the metaphor is whimsically inverted to apply biological lessons to economics, structural deficits become a hallmark of an aging, perhaps even dying economy.

CONCLUSION

In summary, we found foci forming proteins in S. cerevisiase and S. pombe showed the inverse relationship predicted by the "life on the edge" hypothesis for protein aggregation. We showed that the rate Gln1-GFP aggregates into foci is inversely proportional to the available concentration of glucose, demonstrating the availability of cellular energy affects aggregation rate *in vivo*. Thus we propose extending "the life-on-the-edge hypothesis" to include cellular energy in the estimating protein aggregation propensity. We further showed that severe reduction in glycolytic flux by nutrient depletion and addition of a competitive inhibitor of glycolysis produces many smaller foci per cell, suggesting an energy dependence in the agglomeration of smaller Gln1-GFP aggregates. Extending the energy dependence of aggregation to other proteins would support the generality of the phenomena observed for Gln1-GFP aggregates. Testing for the energy dependent mechanisms of foci formation will be important to understanding mechanism yeast employs to cope with the stress of protein aggregation, with potential application to humans.

METHODS

Growth and microscopy of yeast

Yeast strains had a genetic background of BY4741 [genotype, MATa his3_1 leu2_0 met15_0 ura3_0]. Strains expressing GFP-tagged proteins were obtained from the OpenBiosystems GFP collection. Rich (YPD) medium containing Yeast Extract (1%), Peptone (2%) and glucose (2%) was purchased from Sunrise Sciences. Synthetic complete medium (SC) contained 1x Yeast Nitrogen Base (BD Biosciences/Difco)

without amino acids, synthetic drop-out medium supplement mix or was purchased from Sigma. Cultures were started from by picking from freezer stocks into YPD and growing overnight, before transferring to new media for regrowth. Log phase cells were imaged and prepared for mass spec at an O.D. of approximately 2. Cells treated with 2% 2-deoxyglucose (Sigma) were also log–phase cells at an O.D. of approximately 2. Stationary phase cells were grown a minimum of 48 hours before imaging or lysing for mass spec analysis. All cultures were maintained shaking at 30°C.

Cells were imaged on a Nikon E800 fluorescence microscope with Photometrix Coolsnap CCD camera under oil immersion at 100x magnification. DIC images and fluorescent images in the GFP channels were collected using standard filter sets. Cell lysate was imaged on a Nikon TE2000-E with a Photometrics Cascade II camera at 60x magnification. Images were processed using Nikon Elements AR.

Analysis of Gln1-GFP foci composition

Methods for purification and mass spectrometry analysis of Gln1-GFP foci are listed in Chapter 3 in detail.

Imaging flow cytometry for automating Gln1-GFP assays

Methods for purification and mass spectrometry analysis of Gln1-GFP foci are listed in Chapter 3 in detail. In short, cells in early log phase (~2O.D./ml) were spun down and resuspended in SC with glucose concentrations ranging from 0 to 2% (again at ~1O.D./ml). At 30 minute or 1 hour intervals, 300µl of culture was removed and fixed by

incubation with 4% formaldehyde at room temperature for 60 min, and washed with PBS before storing at 4°C. Cells were imaged on an Amnis ImageStream at 60x in brightfield and GFP channels. For all replicates, 50,000 cell images were at a flow rate of 40mm/s and a flow width of 7mm collected. Images were analyzed using custom templates in Amnis IDEAS 4.0 software. The percent of cells with foci was calculated as the number of cells with at least 30% of GFP fluorescence intensity in less than ~1μm^2 . The percent penetrance data was graphed to produce onset curves which where fitted with an allosteric sigmoidal equation to calculate the onset rate of foci formation. The time value at fifty percent max penetrance was plotted against glucose concentration to determine energy dependence of puncta formation.

2-deoxy glucose treatment

Cells in late log phase (~OD 2) were treated with 2% 2-deoxyglucose (Sigma) for 2h shaking at 30C and imaged as described above.

Author's Contributions

S. Pombe library re-analysis, TANGO score calculations, and abundance data analysis was the work of Mark Tsechansky. Glucose reduction experiment was the work of the author. 2-deoxy glucose experiments were the work of Mark Tsechansky and the author.

SUPPORTING INFORMATION

Systematic Name	Standard Name
YAL003W	EFB1
YBL027W	RPL19B
YBL087C	RPL23A
YBL092W	RPL32
YDL136W	RPL35B
YDL191W	RPL35A
YDR064W	RPS13
YDR382W	RPP2B
YDR385W	EFT2
YER056C-A	RPL34A
YER074W	RPS24A
YER117W	RPL23B
YGL076C	RPL7A
YGR192C	TDH3
YIL052C	RPL34B
YIL069C	RPS24B
YJR070C	LIA1
YJR105W	ADO1
YJR123W	RPS5
YKL056C	TMA19
YKL117W	SBA1
YLR029C	RPL15A
YLR325C	RPL38
YML026C	RPS18B
YMR121C	RPL15B
YNL301C	RPL18B
YNL302C	RPS19B
YOL040C	RPS15
YOL121C	RPS19A
YOR133W	EFT1
YOR310C	NOP58
YOR317W	FAA1
YOR332W	VMA4
YPL131W	RPL5
YPL198W	RPL7B

Table S4.1. Yeast genes of the orthologs that became more insoluble in both yeast and worm with age.

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Chapter 5: Discussion

In the following sections discuss potential for widespread protein aggregation to influence cellular senescence, speculate on general models for how complex quaternary structures form in the first place, and why they may be inevitable, and future directions for research.

General protein aggregation as a mechanism for senescence in quiescent cells

Senescence is generally defined as the gradual decline in an organism's ability carry out normal functions. Typically this correlates with increasing age. However, aging is not inherently linked to senescence, and lifespans can vary greatly between closely related species [183] and even within the same species [184], [185]. Moreover, the rate of deterioration is not necessarily linked with the rate of aging, but can be altered by genetic [186], chemical [131], and dietary interventions [181].

To state these relationships thermodynamically, aging is the accumulation of entropy within cellular systems driving them towards equilibrium. However, organisms are not strictly subject to an inexorable increase in entropy as described by the second law of thermodynamics since they are not closed systems, but readily open to acquire free energy from and dump waste into their environments [187]. The question, "why then do organisms age?" still looms as a major question in biology.

Protein aggregation may be one of the answers. While cells may by capable of acquiring free energy from their environment, they do not necessarily possess the means to use it to repair all types of damage. In the case of protein aggregates, degradation machinery is often incapable of overcoming the highly stable bonds formed [188]. Dividing cells, whether bacterial or of developing multi-cellular organisms, can simply divide away from the damage, diluting it at every cell division [189] or partitioning the damage into one of the two resulting cells, creating an old cell and a young cell [190][87]. Quiescent cells by definition lack this damage disposal mechanism, which may explain the higher incidence of protein aggregation diseases in quiescent cells (e.g., nerve and lens cells) [91], [191]. Furthermore, the mechanisms to control proteostasis decline with cellular age, reducing cell ability to prevent [153]and remove aggregates [123][192]. General protein aggregation increases the onset toxicity of disease causing protein aggregates. Thus an age dependent increase in general aggregation in quiescent cells could contribute to senescence and cell death.

Of the few organisms that show negligible senescence, i.e. no apparent age-related decline, many continue to grow for their entire lifetimes [193]. Thus continued cell division may be a requisite mechanism for escaping senescence from protein aggregation.

This chapter has, in part, been excised and reworked from a review by O'Connell et al. 2012 [107].

A case study in ambiguity: Are CTP synthase fibers cytoskeletal elements or bacterial sickle cell disease?

The evolutionary conservation of CTP synthase filaments suggests that filament assembly of CTP synthase may provide a biologically useful purpose for cells, such as enhancing enzymatic regulation or improving CTP synthesis efficiency via compartmentalization, or even forming new cytoskeletal elements. This hypothesis may be (weakly) supported by the observation that yeast CTP synthase isozymes Ura7p and Ura8p co-localize to the same filaments [41]. However, enzymatic activity has yet to be shown for such filaments. Direct functional evidence for CTP synthase filaments is currently limited to the observation that CTP synthase helps determine the *C. crescentus* cell curvature, as well as to the observations that the assembly and characteristics of CTP synthase filaments seem to vary according to cell cycle or nutrient availability. However, as other model organisms in which CTP synthase filaments are found do not exhibit curved cell structures, the roles of filaments in those organisms are unresolved. Notably, filament formation and enzymatic roles are at least partially separable—Ingerson-Mahar *et al.* separated the filament-forming properties of CTP synthase from its enzymatic activity by showing that catalytically inactive synthetase domain mutants retained the ability to form filaments in *C. crescentus* [44].

Another protein oligomerization event known to disrupt cell shape is interesting to consider in this context—that of sickle cell hemoglobin (HbS), arising from normal hemoglobin via a single glutamatic acid to valine substitution. Upon deoxygenation, HbS tetramers polymerize into rigid fibers capable of distorting red blood cells, impeding their

passage through capillaries. The structure and assembly of deoxy-HbS fibers have been studied extensively by electron microscopy [194–196]. Although individuals with sickle cell disease may have drastically reduced quality of life, the mutated gene has been proven to offer resistance against malaria [197], a life-saving advantage which may explain its prevalence in the population. Deoxy-HbS fibers thus represent a case of pathological aggregation that nonetheless offer a non-obvious yet substantial advantage to individuals carrying the mutation. Hence, it is worth noting that CTP synthase fibers might in fact be generally detrimental, yet have persisted throughout different phyla due to a similar yet undiscovered benefit to the organisms. CTP synthase and sickle cell hemoglobin illustrate the difficulties in distinguishing functional roles from aggregation when studying intracellular fibers.

Are metabolic enzymes intrinsically more likely to self-assemble?

As with the large-scale screens of protein localization, microscopy screens have also identified numerous new yeast prions [198]. Thus, whether assembling bodies for functional reasons, perhaps in response to metabolic cues, or simply aggregating pathologically, a much larger set of proteins than broadly appreciated may assemble into bodies or aggregate *in vivo*, perhaps whenever their abundances exceed tolerated limits [106]. The breadth of these phenomena raises the interesting possibility that perhaps all ordered proteins exhibit some level of self-aggregation or self-assembly. Because metabolic enzymes often exhibit complex quaternary structures and intersubunit allostery for the purpose of regulation, such quaternary structures might be intrinsically be more

susceptible to forming intracellular foci and fibers, simply as a result of a symmetrical arrangement of enzymes, replicating any favorable intersubunit interactions around the structure's axes of symmetry. For example, stacked *E. coli* glutamine synthase dodecamers (dimers of hexamers) present six identical interfaces, one between each pair of the six repeated monomers around the dodecamer-dodecamer interface. Any favorable interaction at one such interface is therefore copied six times around the rings, making—*via* avidity—for a potentially very strong overall interface. Such a mechanism of fiber formation is therefore intrinsically more likely for proteins with complex quaternary structures typical of those found among metabolic enzymes. Figure 1, below, shows a gallery of metabolic enzyme quaternary structures and fibers thought to be formed by such stacking mechanisms.

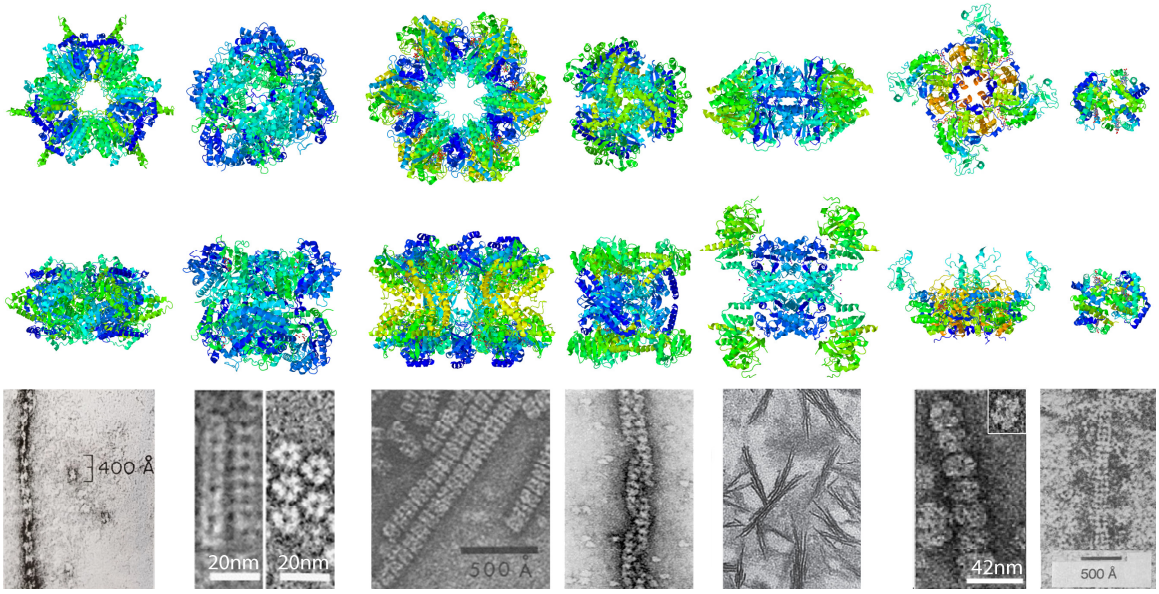

Figure 5-1. A sampling of metabolic enzymes that self-assemble into fibers.

The quaternary structure of each enzyme is illustrated schematically (top row), following 90 degree rotation (middle row), and as visualized by electrion microscopy (bottom row). From left to right, the protein fibers are: rat liver acetyl-CoA carboxylase [199], oat ß-glucosidase [31], *E. coli* glutamine synthetase [32], cow liver glutamate dehydrogenase [34], *Drosophila* CTP synthase (Liu 2010), human type II inosine monophosphate dehydrogenase [44], and human sickle cell mutant hemoglobin [200]. Crystal structures left to right are: *S. coelicolor* acetyl-CoA carboxylase ß-subunit, PDB ID: 1XO6 [201]; wheat ß-glucosidase, PDB ID: 2DGA [202]; *E. coli* glutamine synthetase, PDB ID: 1FY (Gill & Eisenberg 2001); *Clostridium symbiosum* glutamate dehydrogenase, PDB ID: 1BGV [204]; human CTP synthase 2 PDB ID: 3IHL (Moche et al. Unublished data); human type II inosine monophosphate dehydrogenase, PDB ID: 1NF7 (Risal et al. Unpublished data); and human sickle cell hemoglobin, PDB ID: 2HBS [205]. All images created with Jmol.

Given that metabolic enzymes are also often highly expressed and prone to allosteric regulation of alternate conformations, they would seem especially likely cellular candidates for self-assembly into higher order structures. The resulting fibers might provide regulatory functionality—e.g. providing fine tuning of enzymatic output by integrating allosteric interactions across the polymeric interfaces. Such appears to be the case for glutamine synthase, at least on the scale of binding of two homo-pentameric or hexameric rings [80]. Such fibers or assemblies might also provide stability or rigidity, especially as regards the formation of metabolic enzymes into more extensive structures such as crystals, which we might expect to serve as "self-chaperoning" structures, decreasing the likelihood for component proteins to unfold and form aggregates. Indeed, lens crystallins are frequently derived from metabolic enzymes [191]. Similarly, the crystalline cores of peroxisomes, or mitochondrial glutamate dehydrogenase fibers, are cases where homo-oligomeric enzymes are highly concentrated in stressful environments.

Protein aggregation as an evolutionary compromise

As we have discussed, the formation of intracellular protein aggregates is widespread and frequently functional. Therefore, this would seem to be a phenomenon that is not highly selected against, and that may actually be beneficial. However, phylogenetic analyses of multiple proteins suggest that a negative correlation exists between the rate of sequence change and the level of expression [169], [206], which has been interpreted to mean that mistranslation of a highly expressed protein is more likely to lead to dysfunctional aggregation than the mistranslation of scarcer proteins. Thus, despite the widespread existence of protein aggregates, the entire proteome is constantly under selection to avoid aggregates. The resolution of this conundrum would seem to be that aggregates are largely unavoidable and that functional organization around aggregates is an evolutionary compromise.

This begs the question of why aggregates are unavoidable given that evolutionary optimization often grinds genotypes and phenotypes to a very fine degree. The answer must lie in the realm of physical principles that not even evolution can refine, and as inferred above, sickle cell hemoglobin remains one of the most illustrative examples. A single mutation that occurred independently several times within a relatively short span of evolutionary time [207] leads to polymer formation, which can have the beneficial consequence of increased tolerance to malarial infection. We would argue that such mutations are largely unavoidable, especially in oligomeric proteins that by definition have the opportunity to form multiple, geometrically repeated contact points.

Because oligomers are often allosterically regulated, and thus by definition assume multiple conformations, there may be unique opportunities for the formation of new mutational contact points. In many cases, new aggregates will be deleterious, as with Alzheimer's and other prion-based diseases, and the sequence of the protein will eventually be constrained by evolution in a concentration-dependent manner. In some cases, the aggregates will be neutral, and quaternary structures will form that have the opportunity to eventually benefit the cell—possibly through the adoption of a new regulatory functionality, as in the case of acetyl-coA carboxylase. CTP synthase remains a tantalizing example of a higher-order structure whose purpose either remains undiscovered, or that is truly near neutrality, and exists solely as a decoration in the cell.

Future work

Finally an exciting extension of the work described in this dissertation would be an expanded investigation for conservation of protein aggregation with age to more evolutionarily distant organisms. Assaying in organisms that show negligible senescence would be particularly fascinating. These organisms would be excellent model systems to test if the rate of protein aggregate accumulation varies in proportion to lifespan and how protein aggregates are partitioned to prevent accumulation.

Additionally, Characterization of foci forming proteins would also be valuable. Repeating the colocalization tests for known cellular structures on the 178 untested proteins would be quite informative. subdifraction localization of proteins in foci would likewise, be illuminating. Finally, checking the effects of the GFPS35T tag used in the

GFP library on the solubility partitioning would establish the implied link between foci formation and an observed shifts the insoluble phase, that we frequently see correlated.

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